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PCT/NZ2005/000052

CERTIFICATE

This certificate is issued in support of an application for Patent registration in a country outside New Zealand pursuant to the Patents Act 1953 and the Regulations thereunder.

I hereby certify that annexed is a true copy of the Provisional Specification as filed on 28 January 2005 with an application for Letters Patent number 537941 made by Nicolai Vladimirovich Bovin; Lissa Gwyneth Gilliver; Stephen Michael Henry and Elena Yurievna.

I further certify that pursuant to a claim under Section 24(1) of the Patents Act 1953, a direction was given that the application proceed in the name of KIWI INGENUITY LIMITED.

Dated 3 May 2005.

Neville Harris
Commissioner of Patents, Trade Marks and Designs

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537941

Patents Form No. 4

Our Ref: PS223902

Patents Act 1953

PROVISIONAL SPECIFICATION

SYNTHETIC MEMBRANE ANCHORS

We, **Nicolai Vladimirovich BOVIN**, a Russian citizen of 117437 Moscow, Artsimovicha st. 11-181, Russian Federation; **Lissa Gwyneth GILLIVER**, a New Zealand citizen of 134c Church Street, Onehunga, Auckland, New Zealand; **Stephen Michael HENRY**, a New Zealand citizen of 18 Gracechurch Drive, Howick, Auckland, New Zealand; and **Elena YURIEVNA**, a Russian citizen of 117218, Novocheremushkinkaya str. 21-1-26, Moscow, Russian Federation; do hereby declare this invention to be described in the following statement:

PT044261292

100525493_1.RTF

SYNTHETIC MEMBRANE ANCHORS

FIELD OF INVENTION

The invention relates to synthetic molecules that spontaneously and stably incorporate into lipid bi-layers, including cell membranes. Particularly, although not exclusively, the invention relates to the use of these molecules as synthetic membrane anchors or synthetic molecule constructs to effect qualitative and quantitative changes in the expression of cell surface antigens.

BACKGROUND

Cell surface antigens mediate a range of interactions between cells and their environment. These interactions include cell-cell interactions, cell-surface interactions and cell-solute interactions. Cell surface antigens also mediate intra-cellular signalling.

Cells are characterised by qualitative and quantitative differences in the cell surface antigens expressed. Qualitative and quantitative changes in the cell surface antigens expressed alter both cell function (mode of action) and cell functionality (action served).

Being able to effect qualitative and/or quantitative changes in the surface antigens expressed by a cell has diagnostic and therapeutic value.

Cells exist in an aqueous environment. The cell membrane is a lipid bilayer that serves as a semi-permeable barrier between the cytoplasm of the cell and this aqueous environment.

Localising antigens to the cell surface may be achieved by the use of glycolipids as membrane anchors. The natural occurrence of cell surface antigens localised to the cell surface by means of glycolipid membrane anchors is well known.

Isolation of glycolipid-linked antigens and their incorporation into cell membranes to alter the characteristics of a cell has been reported. More recently the preparation of exogenously prepared glycolipid-linked antigens has been reported.

In all these reports the methods include the isolation of a glycolipid or glycolipid-linked antigen from a biological source. The isolation of glycolipids or glycolipid-linked antigens from biological sources is costly, variable and isolatable amounts are often limited.

Obtaining reagents from zoological sources for diagnostic or therapeutic use is problematic where the reagent or its derivative is to be administered to an individual or species of organism different from the source of the reagent. The problem is particularly acute when administration

of the reagent or its derivative to a human subject is contemplated.

Synthetic molecules for which the risk of contamination with zoo-pathogenic agents can be excluded are therefore preferred. Synthetic counterparts for naturally occurring glycolipids and synthetic neo-glycolipids have been reported.

Glycolipids are able to spontaneously and stably incorporate into a lipid bi-layer from an aqueous environment. However, the utility of glycolipid-linked antigens for diagnostic or therapeutic purposes is limited to those glycolipid-linked antigens that will form a solution in saline.

Organic solvents and/or detergents used to facilitate the solubilization of glycolipid-linked antigens in saline must be biocompatible. Solvents and detergents must often be excluded or quickly removed as they can be damaging to some cell membranes. Damage to cell membranes is to be avoided especially where the supply of cells is limited, e.g. embryos.

Removal of solvents and detergents is also required if the preparation is to be administered to an individual as a diagnostic or therapeutic preparation. The removal of solvents or detergents from such preparations can be problematic.

There exists a need for water soluble synthetic molecules that are functionally equivalent to naturally occurring glycolipids and glycolipid-linked antigens in respect of their ability to spontaneously and stably incorporate into lipid bi-layers, including cell membranes.

Providing such synthetic molecules obviates the limitations of glycolipids and glycolipid-linked antigens isolated from zoological sources and facilitates being able to effect qualitative and/or quantitative changes in the surface antigens expressed by a cell by allowing for the use of organic solvents and/or detergents to be excluded.

It is an object of this invention to provide such synthetic molecules and a method for their preparation. It is a further object of this invention to provide diagnostic and therapeutic methods employing the use of such synthetic molecules. The preceding objects are to be read disjunctively with the object to at least provide the public with a useful choice.

STATEMENTS OF INVENTION

In a **first** aspect the invention may broadly be said to consist in a molecule for use as a synthetic membrane anchor of the structure R-S_2-L where:

R is biotin or a chemically reactive functional group;
S_2 is a spacer linking R to L; and
L is a lipid selected from the group consisting of diacyl- and dialkyl-glycerolipids, including glycerophospholipids.

Preferably the molecule is water soluble.

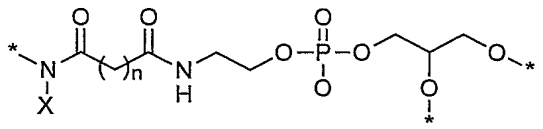
Preferably the molecule spontaneously incorporates into a lipid bi-layer when a solution of the molecule is contacted with the lipid bi-layer. More preferably the molecule stably incorporates into the lipid bilayer.

Preferably R is selected from the group including: biotin, "His tag".

Preferably S_2 is selected from the group including: $-CO(CH_2)_3CO-$, $-CO(CH_2)_4CO-$ (adipate), and $-CO(CH_2)_5CO-$.

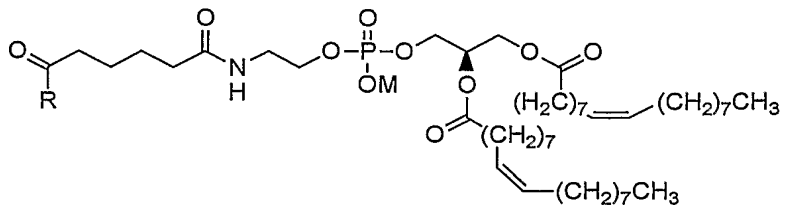
Preferably L is selected from the group consisting of: diacylglycerolipids, phosphatidate, phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, phosphatidyl glycerol, and diphosphatidyl glycerol derived from one or more of *trans*-3-hexadecenoic acid, *cis*-5-hexadecenoic acid, *cis*-7-hexadecenoic acid, *cis*-9-hexadecenoic acid, *cis*-6-octadecenoic acid, *cis*-9-octadecenoic acid, *trans*-9-octadecenoic acid, *trans*-11-octadecenoic acid, *cis*-11-octadecenoic acid, *cis*-11-eicosenoic acid or *cis*-13-docsenoic acid. More preferably L is selected from the group consisting of: 1,2-O-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE), 1,2-O-distearyl-sn-glycero-3-phosphatidylethanolamine (DSPE) and *rac*-1,2-dioleoylglycerol (DOG).

Preferably L is a glycerophospholipid and the molecule includes the substructure:

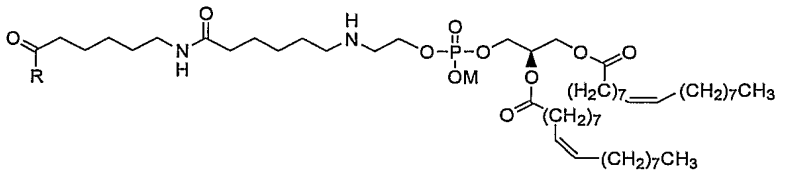


where n = 3 to 5, X is H or C, and * is other than H. Preferably n is 3.

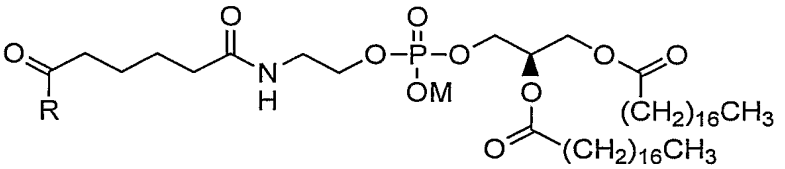
In specific embodiments the molecule has the structure:



designated Ad-DOPE; the structure:



designated sp_1-Ad-DOPE; or the structure:



designated Ad-DSPE.

In a **second** aspect the invention may broadly be said to consist in a synthetic molecule construct of the structure F-S_1-S_2-L where:

F is an antigen (hapten) selected from the group consisting of carbohydrates, proteins, lipids and chemically reactive functional groups;
S_1-S_2 is a spacer linking F to L; and
L is a lipid selected from the group consisting of diacyl- and dialkyl-glycerolipids, including glycerophospholipids.

Preferably the molecule is water soluble.

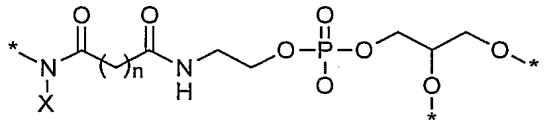
Preferably the molecule spontaneously incorporates into a lipid bi-layer when a solution of the molecule is contacted with the lipid bi-layer. More preferably the molecule stably incorporates into the lipid bilayer.

Preferably F-S_1-S_2-L is covalently bound.

Preferably F is selected from the group consisting of naturally occurring or synthetic glycotopes, antibodies (immunoglobulins), lectins, avidin, and biotin.

Preferably L is selected from the group consisting of: diacylglycerolipids, phosphatidate, phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, phosphatidyl glycerol, and diphosphatidyl glycerol derived from one or more of *trans*-3-hexadecenoic acid, *cis*-5-hexadecenoic acid, *cis*-7-hexadecenoic acid, *cis*-9-hexadecenoic acid, *cis*-6-octadecenoic acid, *cis*-9-octadecenoic acid, *trans*-9-octadecenoic acid, *trans*-11-octadecenoic acid, *cis*-11-octadecenoic acid, *cis*-11-eicosenoic acid or *cis*-13-docsenoic acid. More preferably L is selected from the group consisting of: 1,2-O-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE), 1,2-O-distearyl-sn-glycero-3-phosphatidylethanolamine (DSPE) and *rac*-1,2-dioleoylglycerol (DOG).

Preferably L is a glycerophospholipid and the molecule includes the substructure:



where n = 3 to 5, X is H or C, and * is other than H. Preferably n is 3.

S_1-S_2 is selected to provide a water soluble synthetic molecule construct.

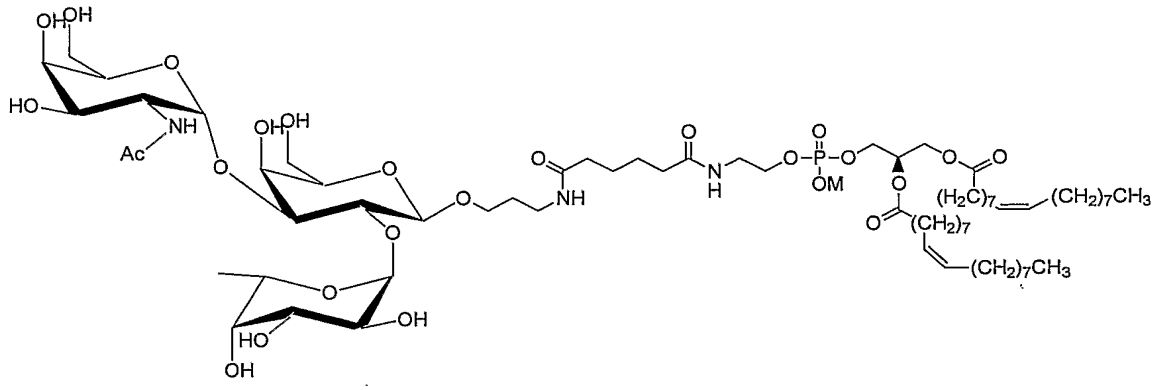
In a first embodiment F is a naturally occurring or synthetic glycotope consisting of three (trisaccharide) or more sugar units. More preferably F is a naturally occurring glycotope selected from the group consisting of lacto-neo-tetraosyl, lactotetraosyl, lacto-nor-hexaosyl, lacto-iso-octaosyl, globoteraosyl, globo-neo-tetraosyl, globopentaosyl, gangliotetraosyl, gangliotriaosyl, gangliopentaosyl, isoglobotriaosyl, isoglobotetraosyl, mucotriaosyl and mucotetraosyl series of oligosaccharides. More preferably F is selected from the group of glycotopes comprising the terminal sugars GalNAcα1-3(Fucα1-2)Galß; Galα1-3Galß; Galß; Galα1-3(Fucα1-2)Galß; NeuAcα2-3Galß; NeuAcα2-6Galß; Fucα1-2Galß; Galß1-4GlcNAcß1-6(Galß1-4GlcNAcß1-3)Galß; Fucα1-2Galß1-4GlcNAcß1-6(Fucα1-2Galß1-4GlcNAcß1-3)Galß; Fucα1-2Galß1-4GlcNAcß1-6(NeuAcα2-3Galß1-4GlcNAcß1-3)Galß; NeuAcα2-3Galß1-4GlcNAcß1-6(NeuAcα2-3Galß1-4GlcNAcß1-3)Galß; Galα1-4Galß1-4Glc; GalNAcß1-3Galα1-4Galß1-4Glc; GalNAcα1-3GalNAcß1-3Galα1-4Galß1-4Glc; or GalNAcß1-3GalNAcß1-3Galα1-4Galß1-4Glc.

When F is an oligosaccharide, L is a glycerophospholipid and S_2 is absent or selected from the group including: $-CO(CH_2)_3CO-$, $-CO(CH_2)_4CO-$ (adipate), $-CO(CH_2)_5CO-$. (e.g. A_1 is *bis*(N-hydroxysuccinimidyl) adipate), preferably S_1 is a C_{3-5}-aminoalkyl selected from the group consisting of: 3-aminopropyl, 4-aminobutyl, or 5-aminopentyl). More preferably S_1 is 3-

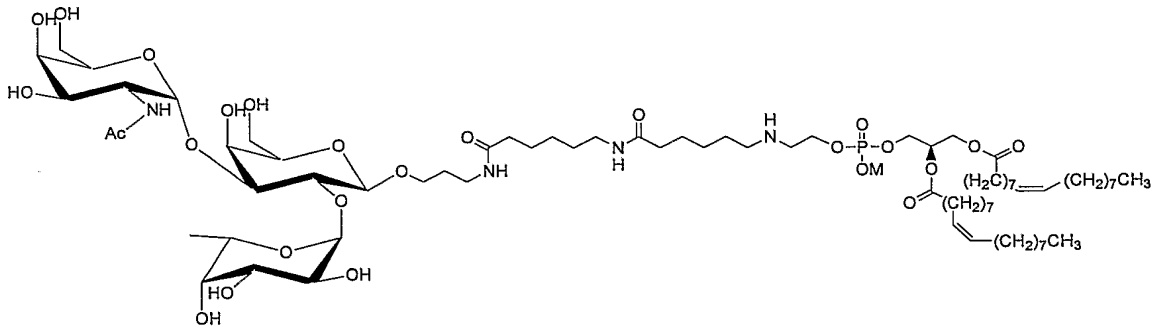
aminopropyl.

When F is an oligosaccharide, L is a glycerolipid and S_2 is -$COCH_2(OCH_2CH_2)nOCH_2CO$- (i.e. A_2 is 4-nitrophenol trifluoroacetate and D is -$ClCOCH_2(OCH_2CH_2)nOCH_2COCl$-), preferably S_1 is a $C_{3\text{-}5}$-aminoalkyl selected from the group consisting of: 3-aminopropyl, 4-aminobutyl, or 5-aminopentyl). More preferably S_1 is 3-aminopropyl.

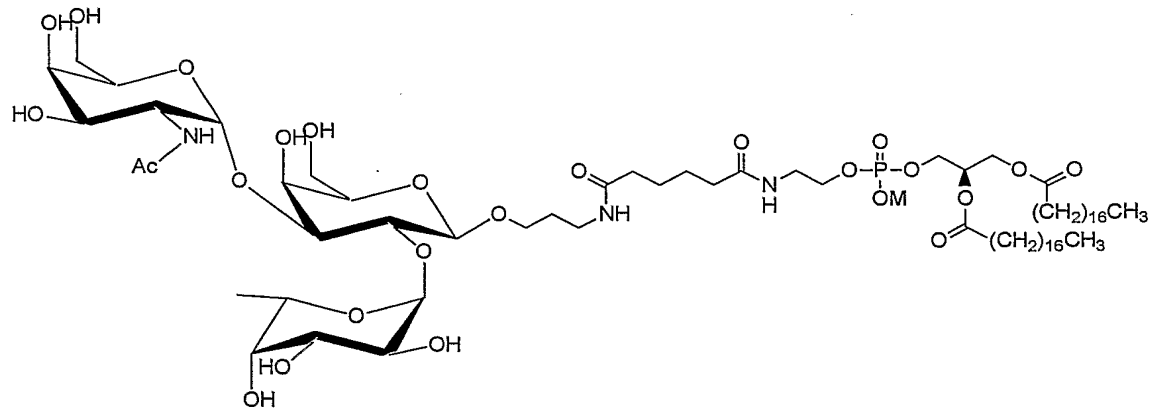
In specific embodiments the water soluble synthetic molecule construct has the structure:



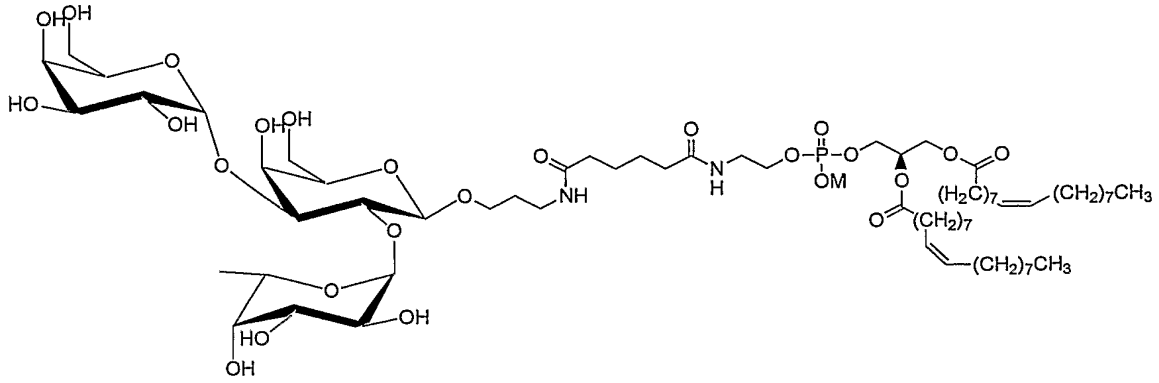
designated A_{tri}-sp-Ad-DOPE (**I**); the structure:



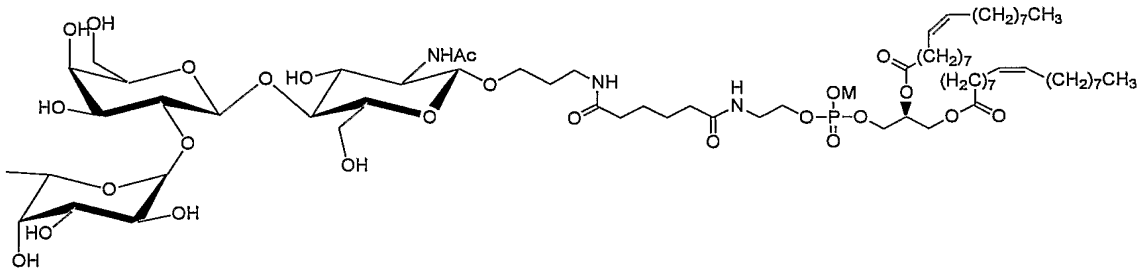
designated A_{tri}-sps_1-Ad-DOPE (**II**); the structure:



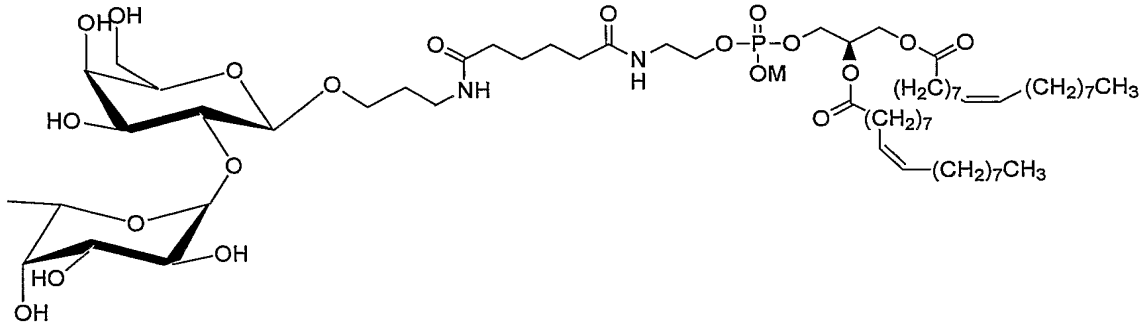
designated A_{tri}-sp-Ad-DSPE (**III**); the structure



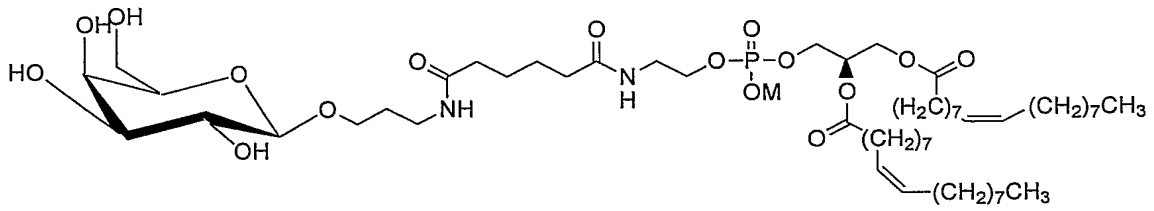
designated B_{tri}-sp-Ad-DOPE (**VI**); the structure:



designated H_{tri}-sp-Ad-DOPE (**VII**); the structure:



designated H_{di}-sp-Ad-DOPE (**VIII**); or the structure:



designated Galβ_1-sp-Ad-DOPE (**IX**).

In a second embodiment F is a molecule that mediates a cell-cell or cell-surface interaction. Preferably F is carbohydrate, protein or lipid with an affinity for a component expressed on a targeted cell or surface. More preferably F has an affinity for a component expressed on epithelial cells or extra-cellular matrices. Most preferably F has an affinity for a component expressed on the epithelial cells or the extra-cellular matrix of the endometrium. Most preferably the component expressed on the epithelial cells or the extra-cellular matrix of the endometrium can be a naturally expressed component or an exogenously incorporated component.

In a third embodiment F is a molecule that mediates a cell-solute interaction. Preferably F is a receptor for a ligand where the presence of the ligand is diagnostic for a pathological condition. More preferably F is an antibody (immunoglobulin) for the ligand (diagnostic molecule), or an antigen for the ligand where the ligand (diagnostic molecule) is an antibody.

In a **third** aspect the invention may broadly be said to consist in a method of preparing a synthetic molecule construct of the structure F-S_1-S_2-L including the steps:

1. Reacting an activator (A_1) with a lipid (L) to provide an activated lipid (A_1-L);
2. Derivatising an antigen (F) to provide a derivatised antigen (F-S_1); and
3. Condensing A_1-L with F-S_1 to provide the molecule;

where:

A_1 is an activator selected from the group including: *bis*(N-hydroxysuccinimidyl), *bis*(4-nitrophenyl), *bis*(pentafluorophenyl), *bis*(pentachlorophenyl) esters of carbodioic acids (C_3 to C_7);
L is a lipid selected from the group consisting of diacyl- and dialkyl-glycerolipids, including glycerophospholipids;
F is an antigen selected from the group consisting of carbohydrates, proteins, lipids, and chemically reactive functional groups; and
S_1-S_2 is a spacer linking F to L where S_1 is selected from the group including: primary aminoalkyl, secondary aliphatic aminoalkyl or primary aromatic amine; and S_2 is absent or selected from the group including: -$CO(CH_2)_3CO$-, -$CO(CH_2)_4CO$- (adipate), and -$CO(CH_2)_5CO$-.

Preferably the molecule is water soluble.

Preferably the molecule spontaneously incorporates into a lipid bi-layer when a solution of the molecule is contacted with the lipid bi-layer. More preferably the molecule stably incorporates into the lipid bilayer.

Preferably F is selected from the group consisting of naturally occurring or synthetic glycotopes, antibodies (immunoglobulins), lectins, avidin, and biotin.

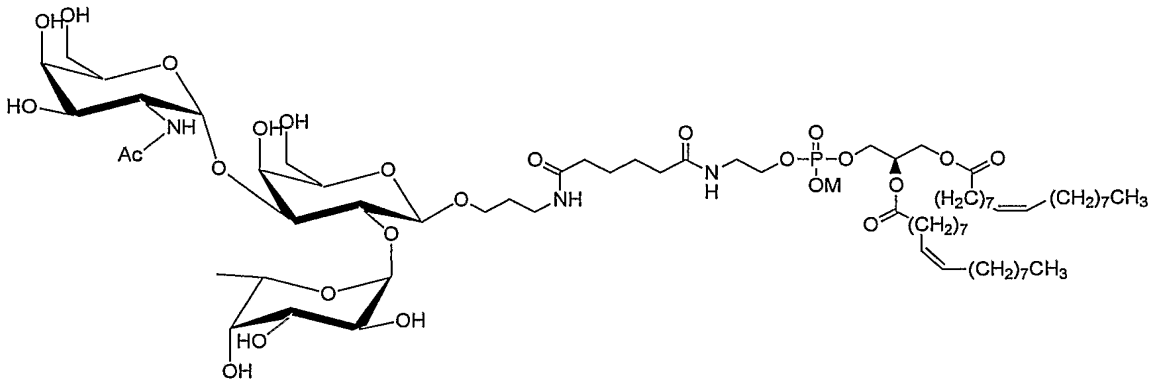
Preferably L is selected from the group consisting of: diacylglycerolipids, phosphatidate, phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, phosphatidyl glycerol, and diphosphatidyl glycerol derived from one or more of *trans*-3-hexadecenoic acid, *cis*-5-hexadecenoic acid, *cis*-7-hexadecenoic acid, *cis*-9-hexadecenoic acid, *cis*-6-octadecenoic acid, *cis*-9-octadecenoic acid, *trans*-9-octadecenoic acid, *trans*-11-octadecenoic acid, *cis*-11-octadecenoic acid, *cis*-11-eicosenoic acid or *cis*-13-docsenoic acid. More preferably L is selected from the group consisting of: 1,2-O-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE), 1,2-O-distearyl-sn-glycero-3-phosphatidylethanolamine (DSPE) and *rac*-1,2-dioleoylglycerol (DOG).

S_1 and S_2 are selected to provide a water soluble synthetic molecule construct that spontaneously incorporates into a lipid bi-layer when a solution of the molecule is contacted with the lipid bi-layer.

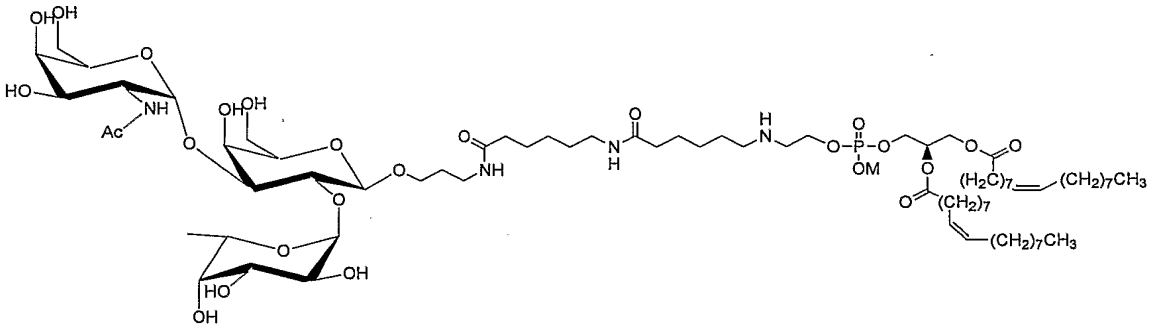
In a first embodiment F is a naturally occurring or synthetic glycotope consisting of three (trisaccharide) or more sugar units (oligosaccharide). More preferably F is a naturally occurring glycotope selected from the group consisting of lacto-neo-tetraosyl, lactotetraosyl, lacto-nor-hexaosyl, lacto-iso-octaosyl, globoteraosyl, globo-neo-tetraosyl, globopentaosyl, gangliotetraosyl, gangliotriaosyl, gangliopentaosyl, isoglobotriaosyl, isoglobotetraosyl, mucotriaosyl and mucotetraosyl series of oligosaccharides. More preferably F is selected from the group of glycotopes comprising the terminal sugars GalNAcα1-3(Fucα1-2)Galß; Galα1-3Galß; Galß; Galα1-3(Fucα1-2)Galß; NeuAcα2-3Galß; NeuAcα2-6Galß; Fucα1-2Galß; Galß1-4GlcNAcß1-6(Galß1-4GlcNAcß1-3)Galß; Fucα1-2Galß1-4GlcNAcß1-6(Fucα1-2Galß1-4GlcNAcß1-3)Galß; Fucα1-2Galß1-4GlcNAcß1-6(NeuAcα2-3Galß1-4GlcNAcß1-3)Galß; NeuAcα2-3Galß1-4GlcNAcß1-6(NeuAcα2-3Galß1-4GlcNAcß1-3)Galß; Galα1-4Galß1-4Glc; GalNAcß1-3Galα1-4Galß1-4Glc; GalNAcα1-3GalNAcß1-3Galα1-4Galß1-4Glc; or GalNAcß1-3GalNAcß1-3Galα1-4Galß1-4Glc.

When F is an oligosaccharide, L is a glycerophospholipid and S_2 is absent or selected from the group including: $-CO(CH_2)_3CO-$, $-CO(CH_2)_4CO-$ (adipate), $-CO(CH_2)_5CO-$. (e.g. A_1 is *bis*(N-hydroxysuccinimidyl) adipate), preferably S_1 is a C_{3-5}-aminoalkyl selected from the group consisting of: 3-aminopropyl, 4-aminobutyl, or 5-aminopentyl). More preferably S_1 is 3-aminopropyl.

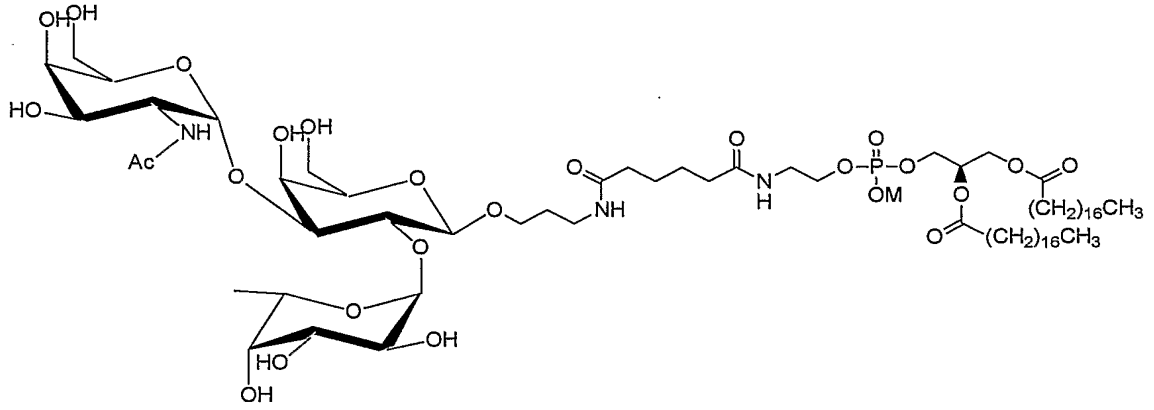
In specific embodiments the water soluble synthetic molecule construct prepared has the structure:



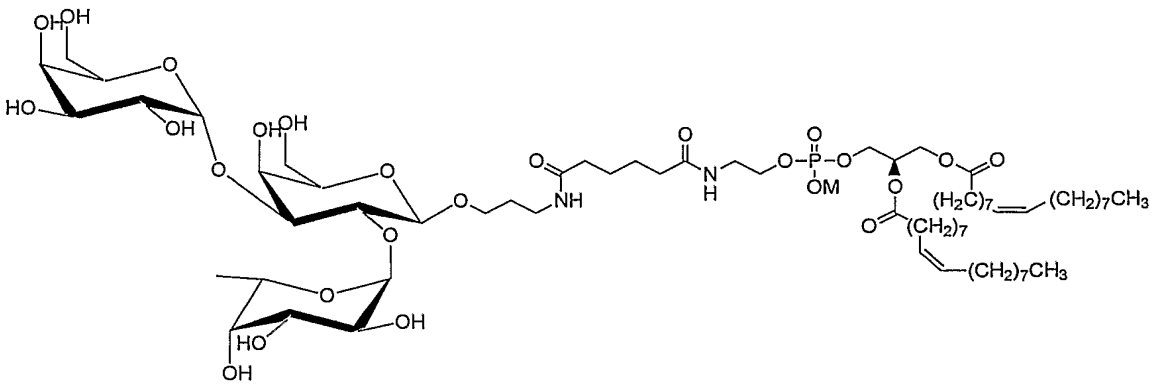
designated A_{tri}-sp-Ad-DOPE (**I**); the structure:



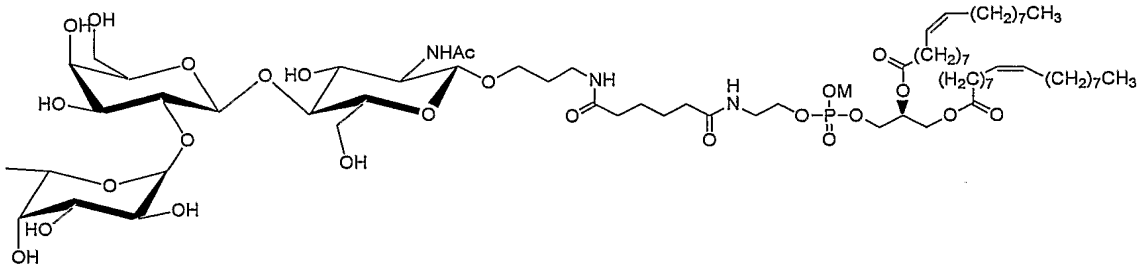
designated A_{tri}-spsp_1-Ad-DOPE (**II**); the structure:



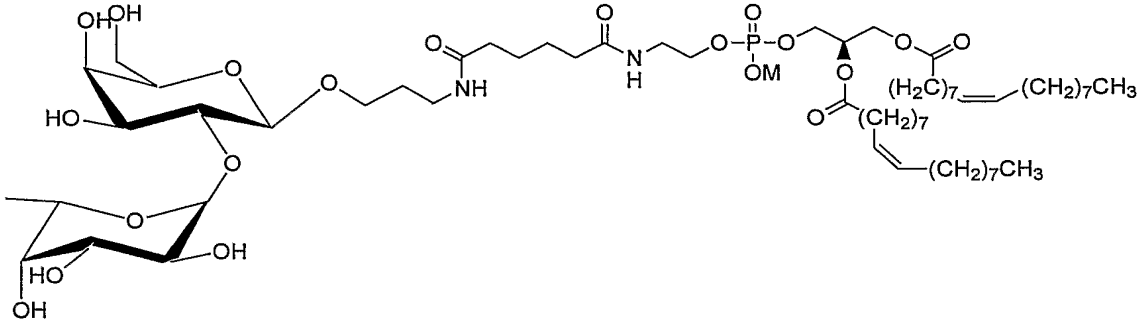
designated A_{tri}-sp-Ad-DSPE (**III**); the structure



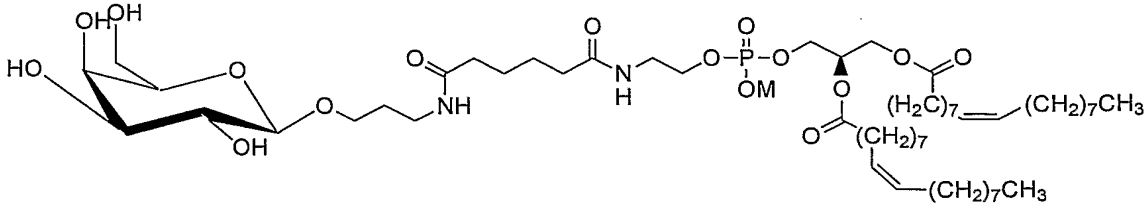
designated B_{tri}-sp-Ad-DOPE (**VI**); the structure:



designated H_{tri}-sp-Ad-DOPE (**VII**); the structure:



designated H_{di}-sp-Ad-DOPE (**VIII**); or the structure:



designated Galβ_{I}-sp-Ad-DOPE (**IX**).

In a second embodiment F is a molecule that mediates a cell-cell or cell-surface interaction. Preferably F is carbohydrate, protein or lipid with an affinity for a component expressed on a

targeted cell or surface. More preferably F has an affinity for a component expressed on epithelial cells or extra-cellular matrix. Most preferably F has an affinity for a component expressed on the epithelial cells or the extra-cellular matrix of the endometrium.

In a third embodiment F is a molecule that mediates a cell-solute interaction. Preferably F is a receptor for a ligand where the presence of the ligand is diagnostic for a pathological condition. More preferably F is an antibody for the ligand (diagnostic molecule), or an antigen for the ligand where the ligand (diagnostic molecule) is an antibody.

In a **fourth** aspect the invention may broadly be said to consist in a water soluble synthetic molecule construct prepared by a method according to the third aspect of the invention.

In a **fifth** aspect the invention may broadly be said to consist in a method of effecting qualitative and/or quantitative changes in the surface antigens expressed by a cell or multi-cellular structure including the step:

1. Contacting a suspension of the cell or multi-cellular structure with a solution of a water soluble synthetic molecule construct according to the second aspect or fourth aspect of the invention for a time and at a temperature sufficient to effect the qualitative and/or quantitative change in the surface antigens expressed by the cell or multi-cellular structure.

Preferably the cell or multi-cellular structure is of human origin.

In a first embodiment the cell is a red blood cell.

In this embodiment preferably F is selected from the group of glycotopes comprising the terminal sugars GalNAcα1-3(Fucα1-2)Galß; Galα1-3Galß; Galß; Galα1-3(Fucα1-2)Galß; NeuAcα2-3Galß; NeuAcα2-6Galß; Fucα1-2Galß; Galß1-4GlcNAcß1-6(Galß1-4GlcNAcß1-3)Galß; Fucα1-2Galß1-4GlcNAcß1-6(Fucα1-2Galß1-4GlcNAcß1-3)Galß; Fucα1-2Galß1-4GlcNAcß1-6(NeuAcα2-3Galß1-4GlcNAcß1-3)Galß; NeuAcα2-3Galß1-4GlcNAcß1-6(NeuAcα2-3Galß1-4GlcNAcß1-3)Galß; Galα1-4Galß1-4Glc; GalNAcß1-3Galα1-4Galß1-4Glc; GalNAcα1-3GalNAcß1-3Galα1-4Galß1-4Glc; or GalNAcß1-3GalNAcß1-3Galα1-4Galß1-4Glc. More preferably F is selected from the group of glycotopes consisting of the oligosaccharides GalNAcα1-3(Fucα1-2)Galß and Galα1-3(Fucα1-2)Galß.

In this embodiment preferably the solution of the water soluble synthetic molecule construct has a concentration of at least 0.05 mg/mL.

In this embodiment preferably the solution of the water soluble synthetic molecule construct is contacted with the red blood cell for at least 1 hour at around 4 °C.

In a second embodiment the multi-cellular structure is an embryo.

In this embodiment preferably F is an attachment molecule where the attachment molecule has an affinity for a component expressed on the epithelial cells or the extra-cellular matrix of the endometrium.

The component expressed on the epithelial cells or the extra-cellular matrix of the endometrium can be a naturally expressed component or an exogenously incorporated component.

In this embodiment preferably the solution of the water soluble synthetic molecule construct is contacted with the multi-cellular structure for at least 1 hour at around 4 °C.

In a third embodiment the cell is red blood cell.

In this embodiment preferably F is a receptor for a ligand where the presence of the ligand is diagnostic for a pathological condition. More preferably F is an antibody (immunoglobulin) for the ligand (diagnostic molecule), or an antigen for the ligand where the ligand (diagnostic molecule) is an antibody.

In a **sixth** aspect the invention may broadly be said to consist in a cell or multi-cellular structure incorporating a molecule for use as a synthetic membrane anchor according to the first aspect of the invention, or a water soluble synthetic molecule construct according to the second or fourth aspect of the invention.

Preferably the cell or multi-cellular structure is of human origin.

In a first embodiment the cell is a red blood cell incorporating a water soluble synthetic molecule construct selected from the group consisting of: A_{tri}-sp-Ad-DOPE (**I**), A_{tri}-spsp_1-Ad-DOPE (**II**), A_{tri}-sp-Ad-DSPE (**III**), B_{tri}-sp-Ad-DOPE (**VI**), H_{tri}-sp-Ad-DOPE (**VII**), H_{di}-sp-Ad-DOPE (**VIII**), and Gal$ß_l$-sp-Ad-DOPE (**IX**).

In a **seventh** aspect the invention may broadly be said to consist in a kit comprising a dried preparation or solution of a molecule for use as a synthetic membrane anchor according to the first aspect of the invention, or a water soluble synthetic molecule construct according to the second or fourth aspect of the invention.

Preferably the molecule for use as a synthetic membrane anchor is selected from the group consisting of: Ad-DOPE, sp_1-Ad-DOPE, Ad-DSPE, Ad-DOPE, and POE_{10}-DOG.

Preferably the water soluble synthetic molecule construct is selected from the group consisting of: A_{tri}-sp-Ad-DOPE (**I**), A_{tri}-spsp$_1$-Ad-DOPE (**II**), A_{tri}-sp-Ad-DSPE (**III**), B_{tri}-sp-Ad-DOPE (**VI**), H_{tri}-sp-Ad-DOPE (**VII**), H_{di}-sp-Ad-DOPE (**VIII**), and Galβ_1-sp-Ad-DOPE (**IX**).

In an **eigth** aspect the invention may broadly be said to consist in a kit comprising a suspension of cells or multi-cellular structures according to the sixth aspect of the invention.

Preferably the cell or multi-cellular structure is of human origin.

In a first embodiment the cells are red blood cells that do not naturally express A- or B-antigen and incorporate a water soluble synthetic molecule construct selected from the group consisting of: A_{tri}-sp-Ad-DOPE (**I**), A_{tri}-spsp$_1$-Ad-DOPE (**II**), A_{tri}-sp-Ad-DSPE (**III**), B_{tri}-sp-Ad-DOPE (**VI**), H_{tri}-sp-Ad-DOPE (**VII**), H_{di}-sp-Ad-DOPE (**VIII**), Galβ_1-sp-Ad-DOPE (**IX**) and A_{tri}-sp-POE$_{10}$-DOG (**X**).

In an **ninth** aspect the invention may broadly be said to consist in a pharmaceutical preparation comprising a dried preparation or solution of a molecule for use as a synthetic membrane anchor according to the first aspect of the invention, or a water soluble synthetic molecule construct according to the second or fifth aspect of the invention.

Preferably the pharmaceutical preparation is in a form for administration by inhalation.

Preferably the pharmaceutical preparation is in a form for administration by injection.

In an **tenth** aspect the invention may broadly be said to consist in a pharmaceutical preparation comprising a suspension of cells or multi-cellular structures according to the sixth aspect of the invention.

Preferably the cells or multi-cellular structures are of human origin.

Preferably the pharmaceutical preparation is in a form for administration by inhalation.

Preferably the pharmaceutical preparation is in a form for administration by injection.

DETAILED DESCRIPTION

The synthetic molecule constructs of the invention spontaneously and stably incorporate into a lipid bi-layer, such as a membrane, when a solution of the molecule is contacted with the lipid bi-layer. Whilst not wishing to be bound by theory it is believed that the insertion into the membrane of the lipid tails of the lipid (L) is thermodynamically favoured. Subsequent disassociation of the synthetic molecule construct from the lipid membrane is believed to be

thermodynamically unfavoured. Surprisingly, the synthetic molecule constructs identified herein have been found to be water soluble.

Accordingly the synthetic molecule constructs of the invention are used to transform cells resulting in qualitative and/or quantitative changes in the surface antigens expressed. It will be recognised that the transformation of cells in accordance with the invention is distinguished from transformation of cells by genetic engineering. The invention provides for phenotypic transformation of cells *without* genetic transformation.

In the context of this description the term "transformation" in reference to cells is used to refer to the insertion or incorporation into the cell membrane of exogenously prepared synthetic molecule constructs thereby effecting qualitative and quantitative changes in the cell surface antigens expressed by the cell.

The synthetic molecule constructs of the invention comprise an antigen (F) linked to a lipid portion (or moiety) (L) via a spacer (S_1-S_2). The synthetic molecule constructs can be prepared by the condensation of a primary aminoalkyl, secondary aliphatic aminoalkyl or primary aromatic amine derivative of the antigen with an activated lipid.

A desired phenotypic transformation may be achieved using the synthetic molecule constructs of the invention in a one step method or a two step method. In the one step method the water soluble synthetic molecule construct (F-S_1-S_2-L) comprises the surface antigen as F.

In the two step method the synthetic molecule construct (F-S_1-S_2-L) comprises an antigen (F) that serves as a functional group to which the surface antigen can be linked following insertion of the synthetic molecule construct into the membrane. The functional group can be a group such as avidin, biotin, a chelator or a chemically reactive functional group.

In accordance with the invention the primary aminoalkyl, secondary aliphatic aminoalkyl or primary aromatic amine and the activator of the lipid are selected to provide a synthetic molecule construct that is water soluble and will spontaneously incorporate into a lipid bi-layer when a solution of the synthetic molecule construct is contacted with the lipid bi-layer.

In the context of this description the phrase "water soluble" means a stable, single phase system is formed when the synthetic molecule construct is contacted with water or saline (such as PBS) in the absence of organic solvents or detergents, and the term "solution" has a corresponding meaning.

In the context of this description the phrase "stably incorporate" means that the synthetic molecule constructs incorporate into the lipid bi-layer or membrane with minimal subsequent exchange between the lipid bi-layer or membrane and the external aqueous environment of

the lipid bi-layer or membrane.

The selection of the primary aminoalkyl, secondary aliphatic aminoalkyl or primary aromatic amine and the activator depends on the physico-chemical properties of the antigen (F) to be linked to the lipid (L).

It will be understood by those skilled in the art that for a non-specific interaction, such as the interaction between a diacyl- or dialkyl-glycerolipid and a membrane, structural and stereo-isomers of naturally occurring lipids can be functionally equivalent. For example, it is contemplated by the inventors that diacylglycerol 2-phosphate could be substituted for phosphatidate (diacylglycerol 3-phosphate). Furthermore it is contemplated by the inventors that the absolute configuration of phosphatidate can be either R or S.

The inventors have determined that to prepare synthetic molecule constructs of the invention where the antigen (F) is an oligosaccharide selected from the group of glycotopes for A-, B- and H-antigens of the ABO blood groups, the primary aminoalkyl, secondary aliphatic aminoalkyl or primary aromatic amine, and the activator should be selected to provide a spacer (S_1-S_2) with a structure according to one of those presented in Table 1.

It will be understood by one skilled in the art that once the structure of the spacer (S_1-S_2) has been determined for a given class of antigens, the same structure of the spacer can be adopted to prepare synthetic molecule constructs of other classes of antigen with similar physico-chemical properties.

For example, the structure of the spacer for synthetic molecule constructs (F-S_1-S_2-L) where F is a glycotope of the A-, B- and H-antigens of the ABO blood groups, may be the structure of the spacer selected to prepare synthetic molecule constructs of other antigens with physico-chemical properties similar to the glycotopes of the A-, B- and H-antigens of the ABO blood groups.

In principle the glycotope of a broad range of blood group related glycolipids or glycoproteins could be made the antigen (F) of the synthetic molecule construct F-S_1-S_2-L where S_1-S_2-L is identical or equivalent to the corresponding portion of the synthetic molecule constructs designated A_{tri}-sp-Ad-DOPE (**I**), A_{tri}-spsp_1-Ad-DOPE (**II**), A_{tri}-sp-Ad-DSPE (**III**), B_{tri}-sp-Ad-DOPE (**VI**), H_{tri}-sp-Ad-DOPE (**VII**), H_{di}-sp-Ad-DOPE (**VIII**), and Galß-sp-Ad-DOPE (**IX**).

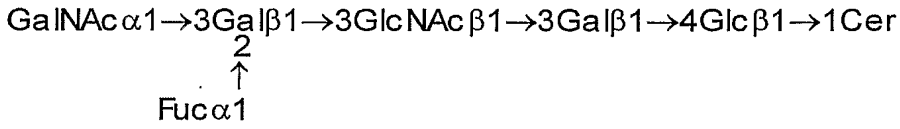
It will be understood by those skilled in the art that the synthetic molecule constructs (F-S_1-S_2-L) of the invention where F is an oligosaccharide may be used as "synthetic glycolipids" and substituted for glycolipids obtained from biological (botanical or zoological) sources.

The structures of known blood group-related glycolipids and glycoproteins (see references) are provided in the following list:

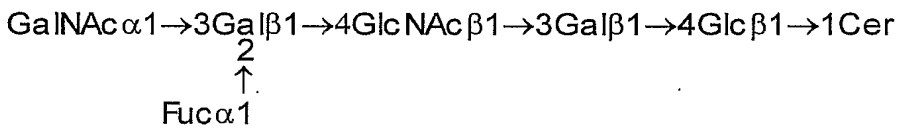
Glycolipids*

(*In general, for almost all examples of A-antigens the terminal A sugar GalNAc can be replaced with the B sugar Gal. Additionally, the lack of either the A or B determinant creates the equivalent H determinant.)

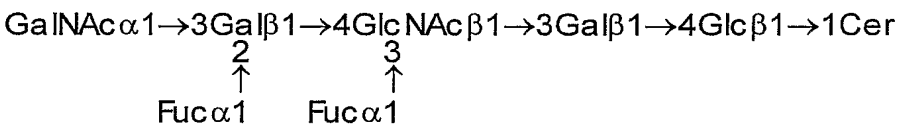
A-6-1



A-6-2



A-7-2 (ALe^y)



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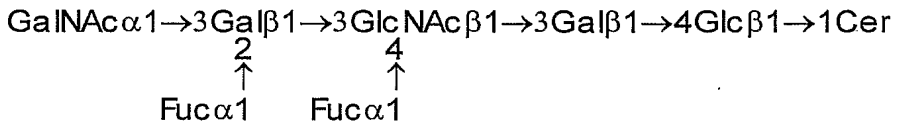
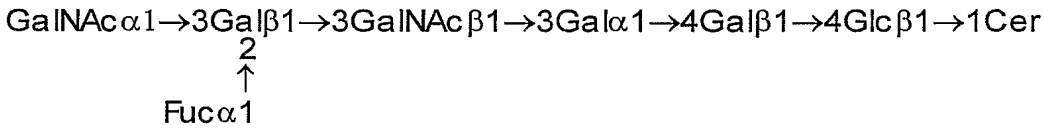
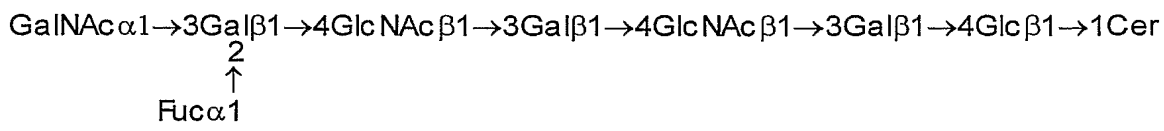


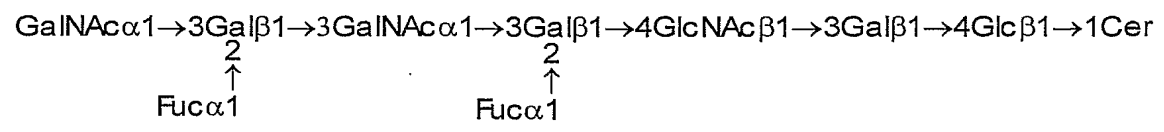
A-7-4



A-8-2



A-9-3



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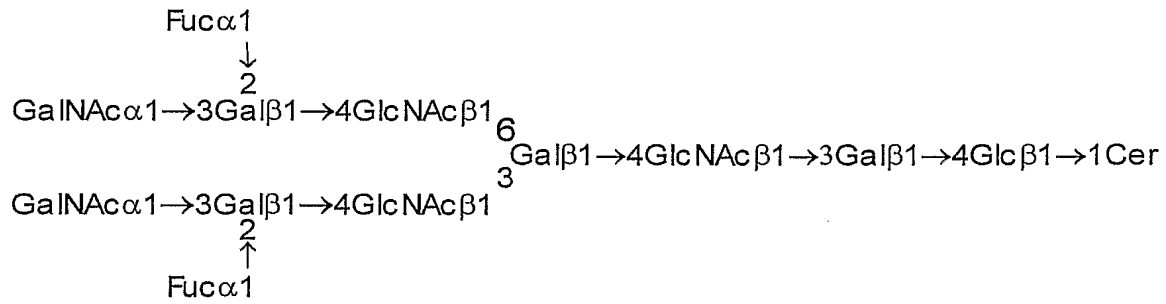

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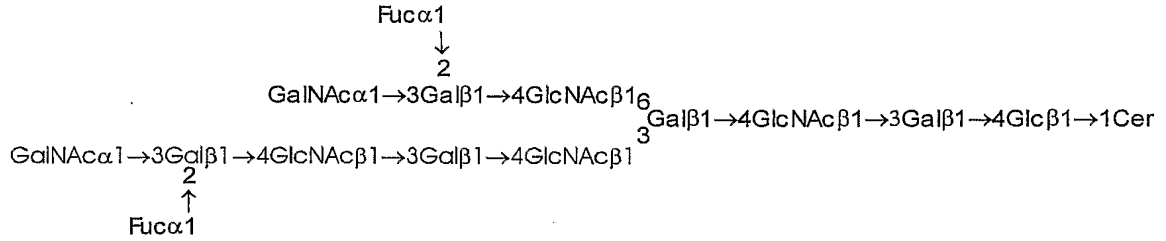
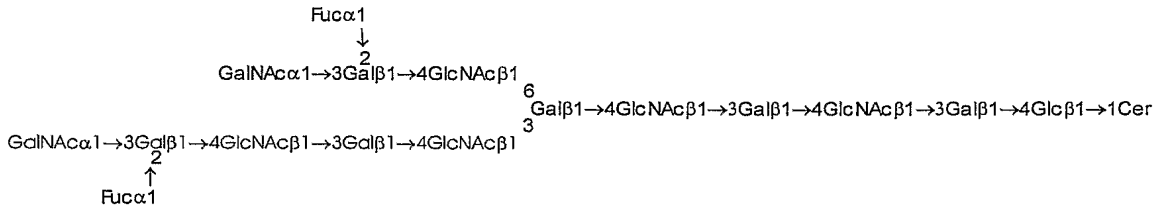


A-16-2



Lactosylceramide

Galβ1→4Glcβ1→1Cer

Hematoside/G_{M3}

NeuAcα2→3Galβ1→4Glcβ1→1Cer

Lactotriaosylceramide

GlcNAcβ1→3Galβ1→4Glcβ1→1Cer

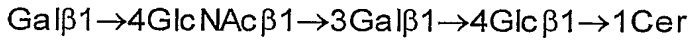
Globotriaosylceramide/P^K

Galα1→4Galβ1→4Glcβ1→1Cer

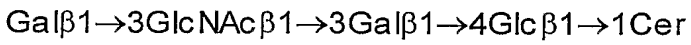
Globoside/P

GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→1Cer

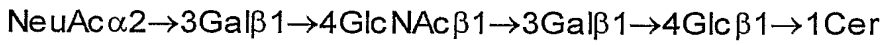
Paragloboside/neolactotetraosylceramide



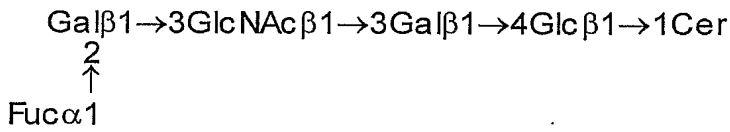
Le^c-4/Lactotetraosylceramide



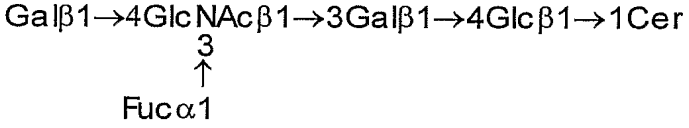
Sialoyl paragloboside/sialoyl neolactotetraosylceramide



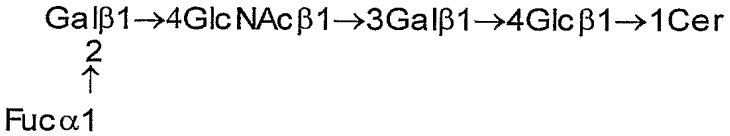
H-5-1



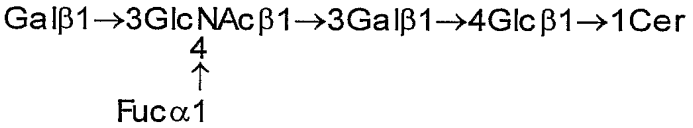
Le^x-5



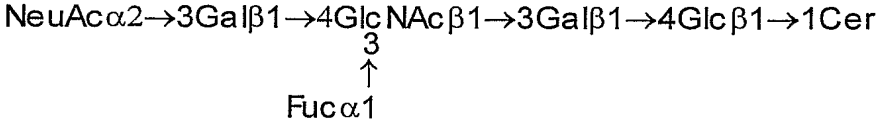
H-5-2



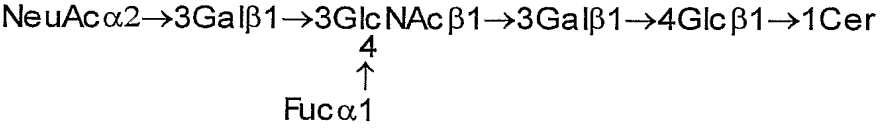
Le^a-5



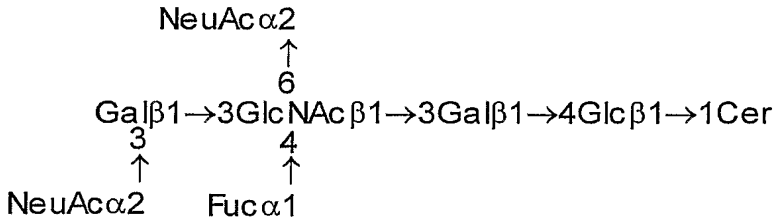
Sialyl Le^x



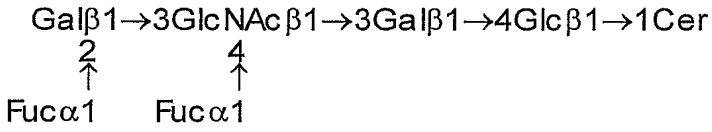
Sialyl Le^a-6/gastrointestinal cancer antigen (GICA or Ca 19-9)



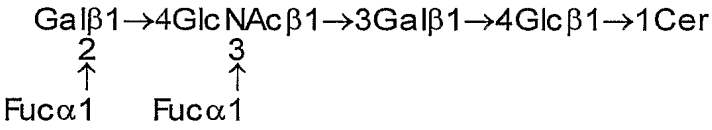
Disialoyl Le^a-7



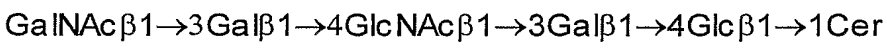
Le^b-6



Le^y-6

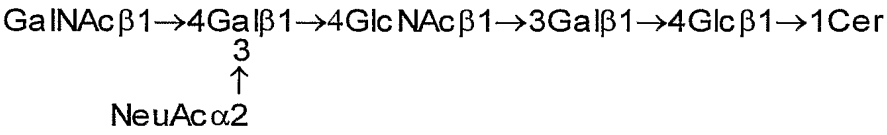


P-like

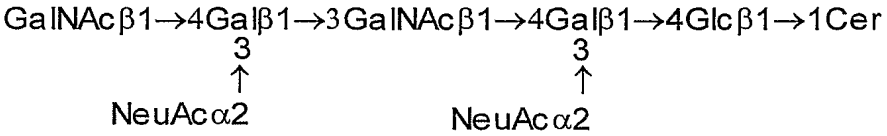


Forssman antigen

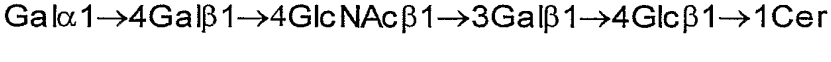
Cad erythrocyte



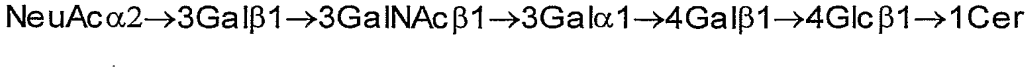
Cad hepato-carcinoma antigen



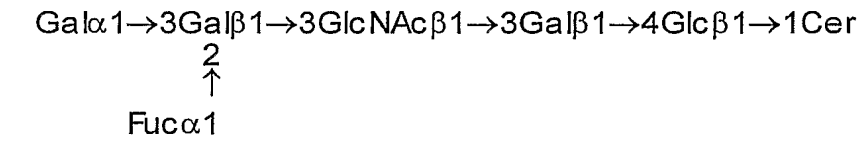
P_1



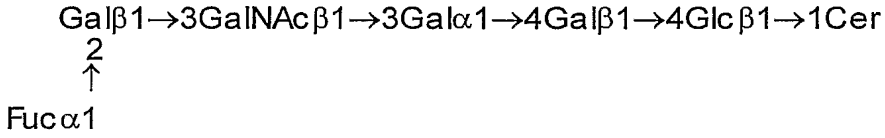
LKE/'GL 7/SSEA-4



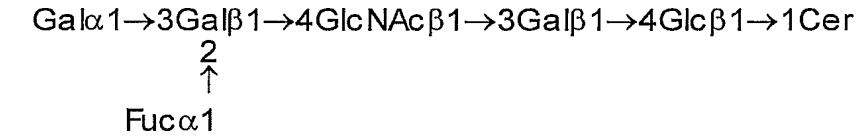
B-6-1



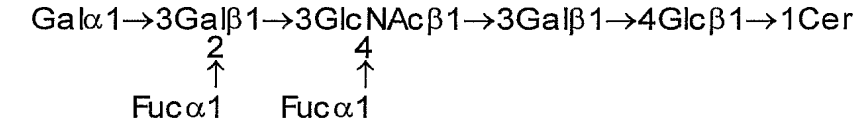
H-6-4



B-6-2



BLe^b-7



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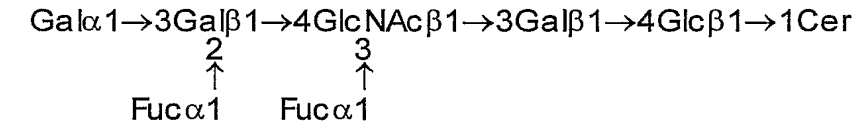
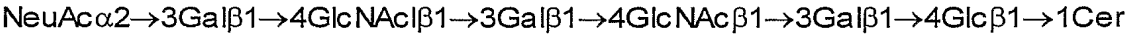
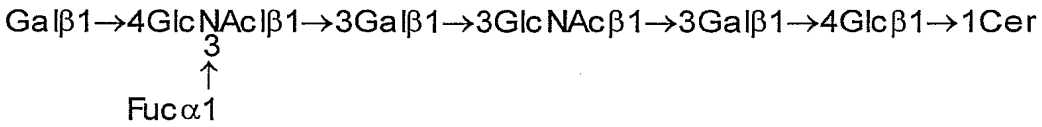


i antigen/lacto-N-*nor*-hexaosylceramide

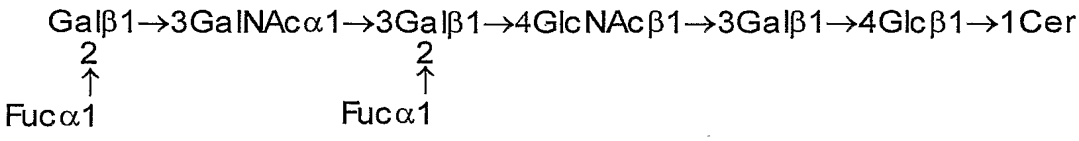
Sialyl-*nor*-hexaosylceramide/sialoyl-lacto-N-*nor*-hexaosylceramide



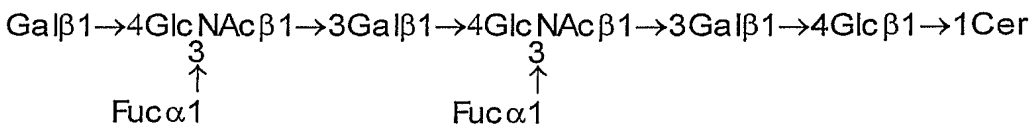
Le^x-7



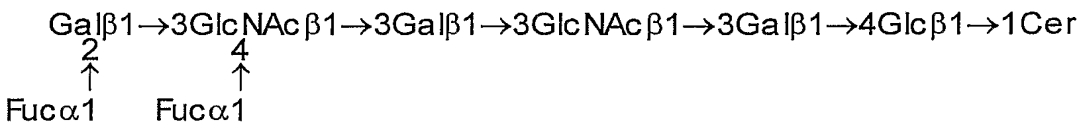
H-8-3



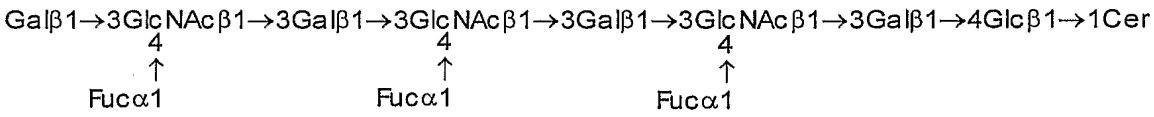
Le^x-8



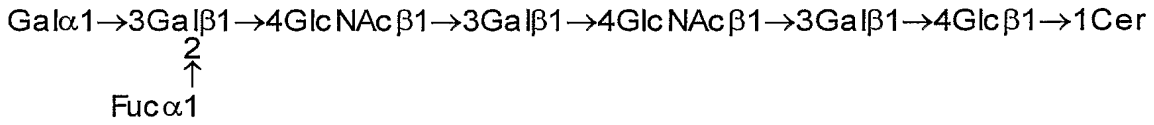
Le^b-8



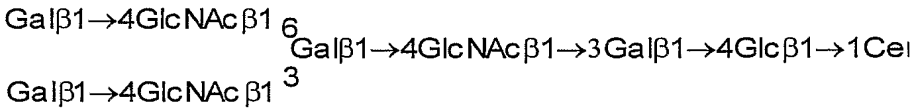
Le^a-11



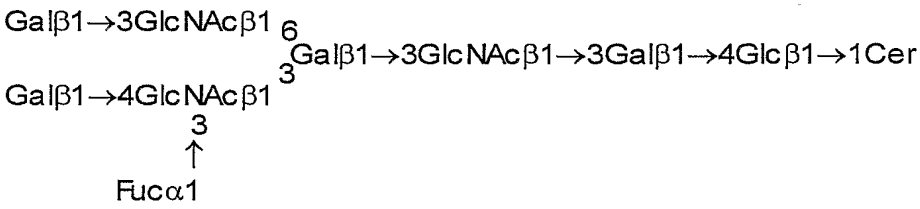
B-8-2



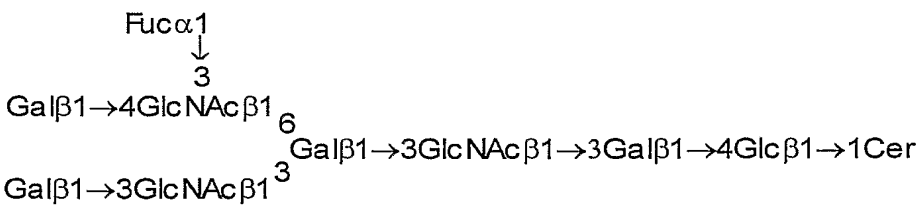
I antigen



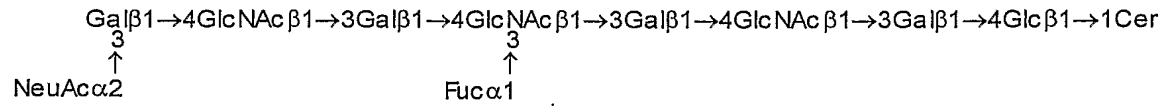
Le^c-9 (fucosylated backbone)



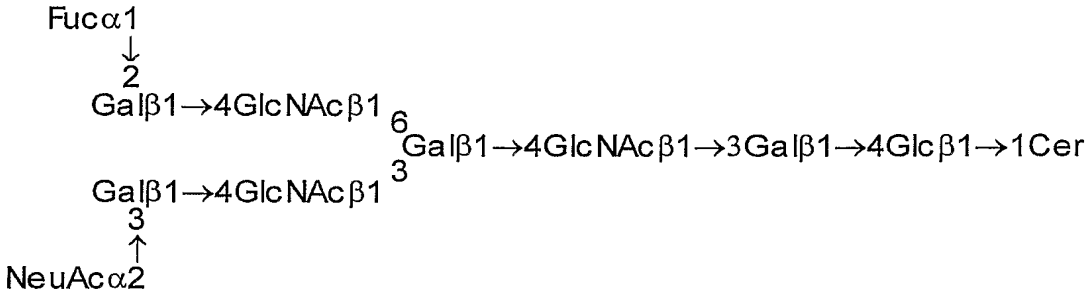
Le^c-9 (fucosylated branch)



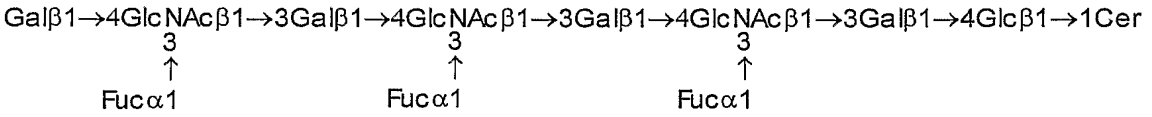
VIM-2



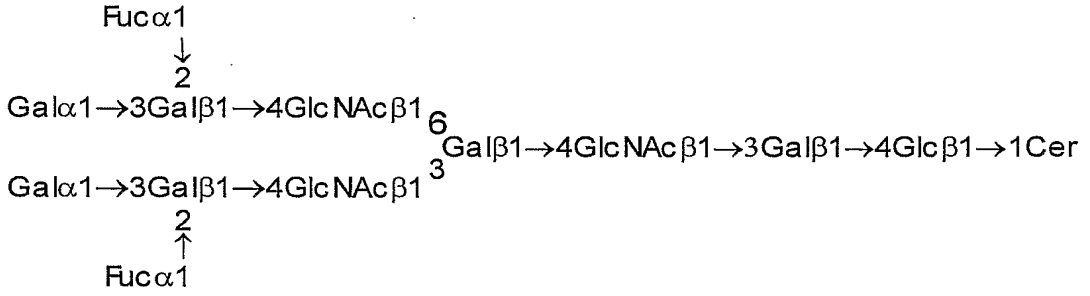
Erythrocyte FI antigen



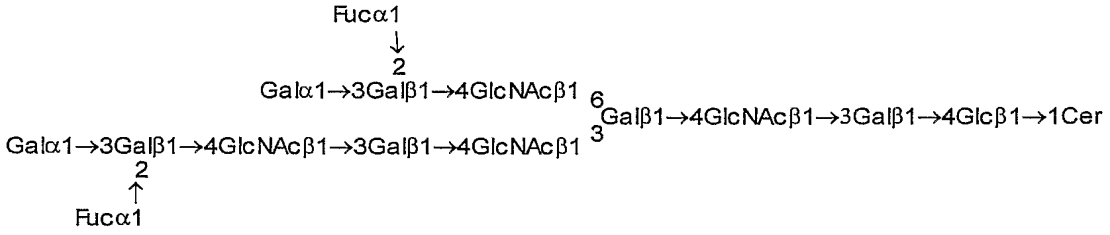
Le^x-11



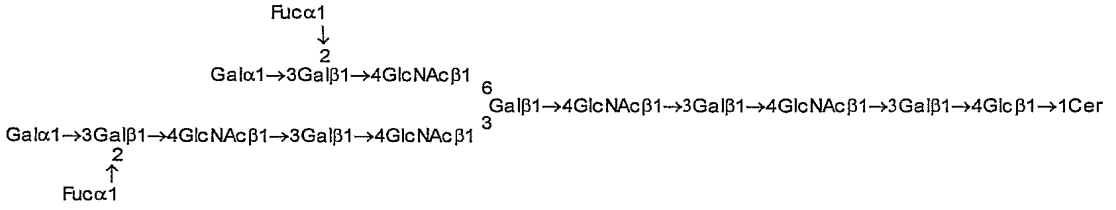
B-12-2



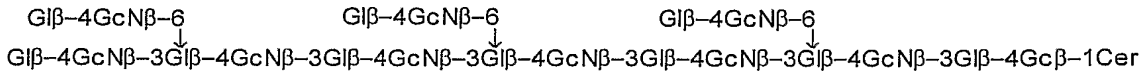
B-14-2



B-16-2

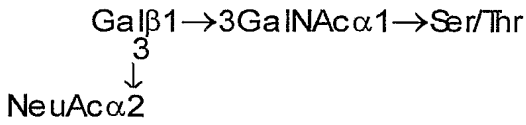


I-active polyglycosylceramide

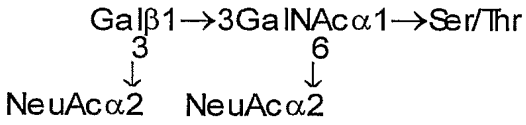


<u>O-linked Glycoproteins</u>

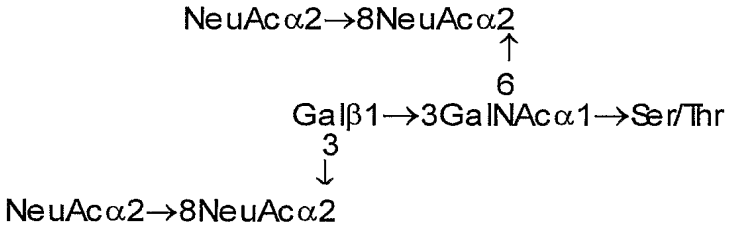
Monosialotrisaccharide



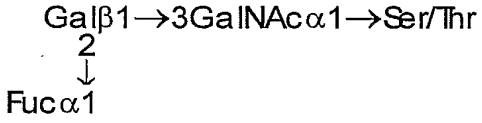
Disialotetrasaccharide



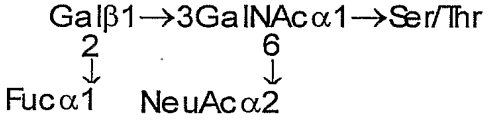
Disialoyl group oligosaccharide



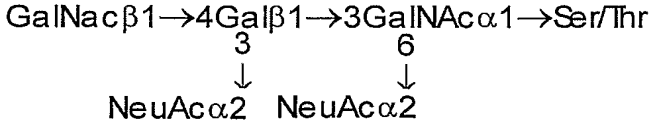
H-active trisaccharide



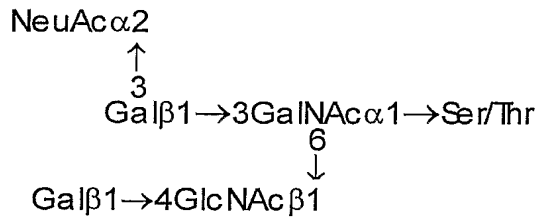
Sialylated H-active tetrasaccharide



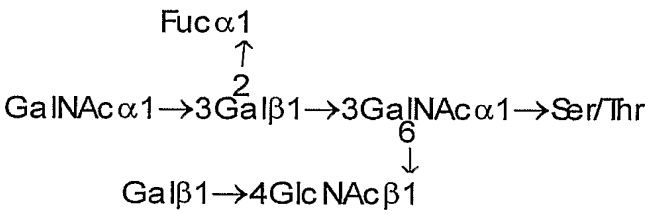
Cad oligosaccharide



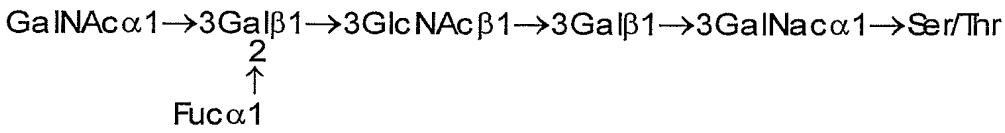
GlcNAc oligosaccharide



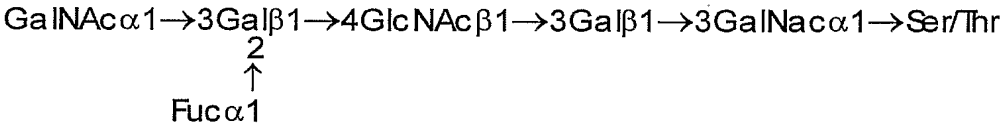
Mucin oligosaccharide/A-active glycoprotein



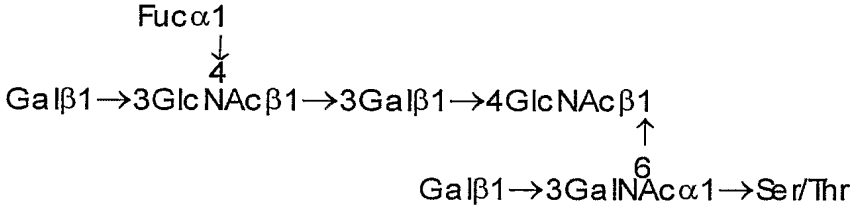
Ovarian cyst A-active glycoprotein-6a



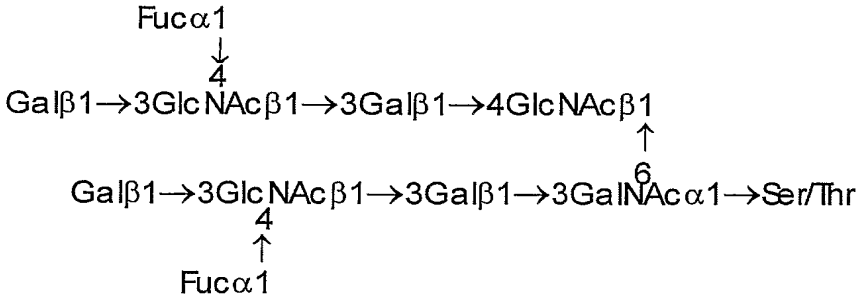
Ovarian cyst A-active glycoprotein-6b



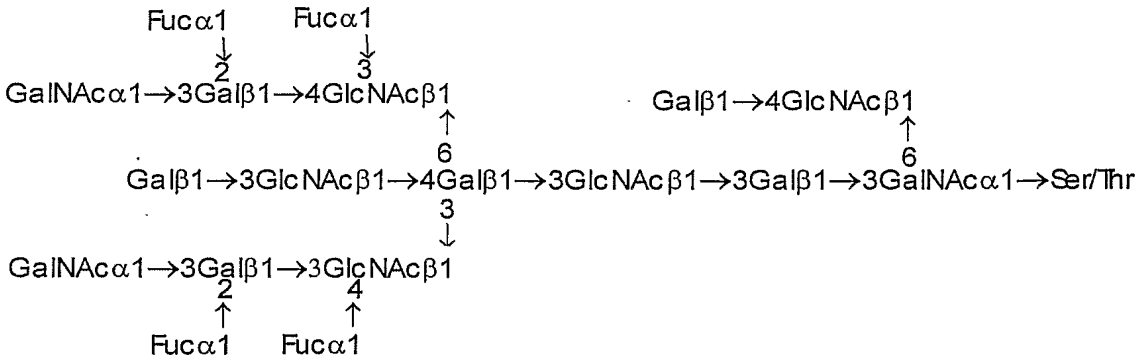
Ovarian cyst Le^a-active glycoprotein-7



Ovarian cyst Le^a-active glycoprotein-10

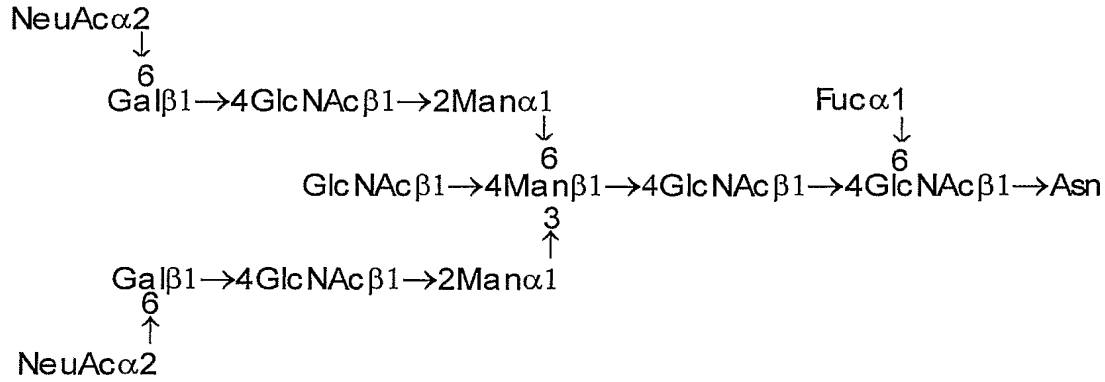


Ovarian cyst A-active glycoprotein-18

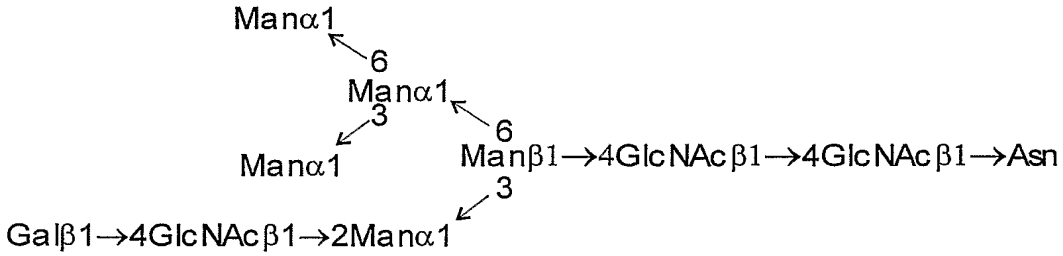


N-linked Glycoproteins

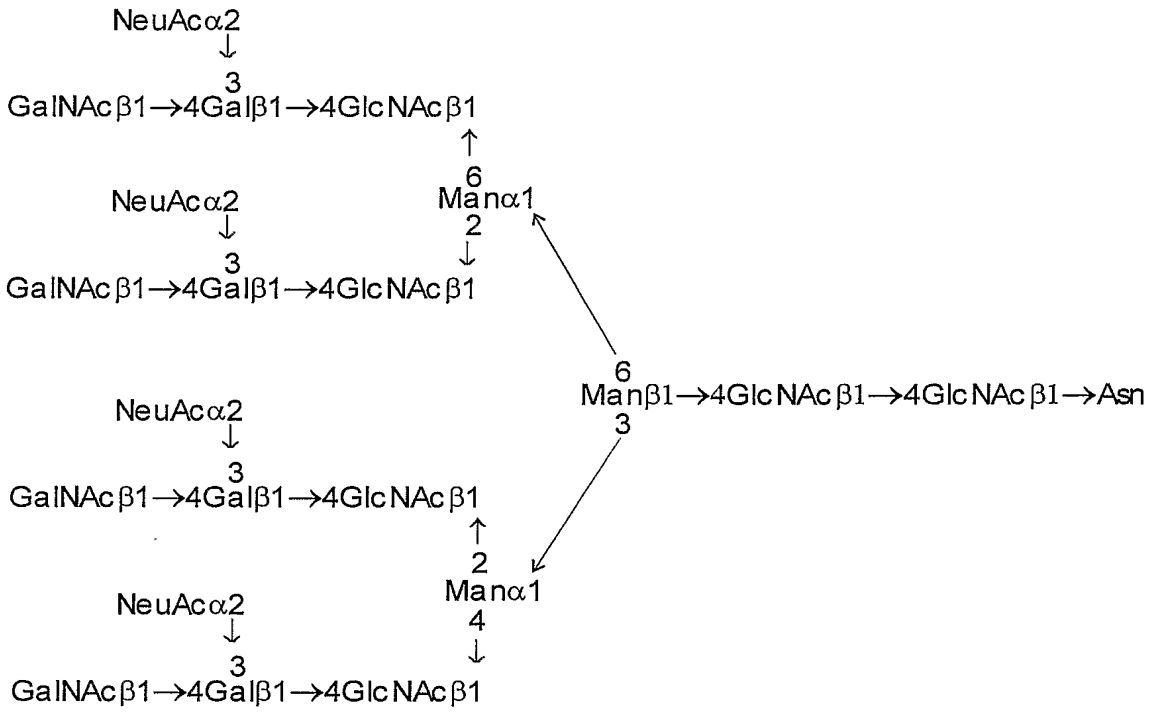
Complex type/Alkali-stable chain



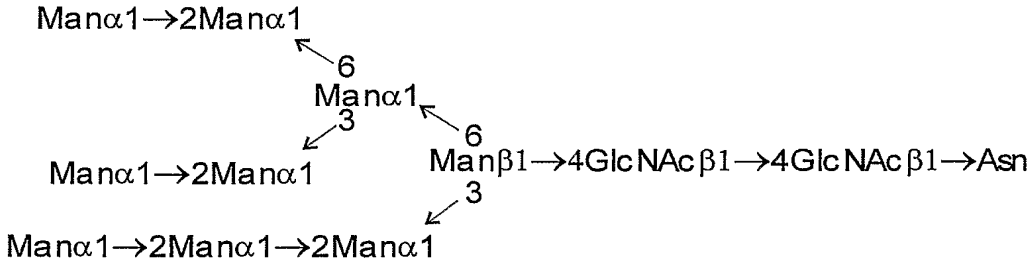
Hybrid type



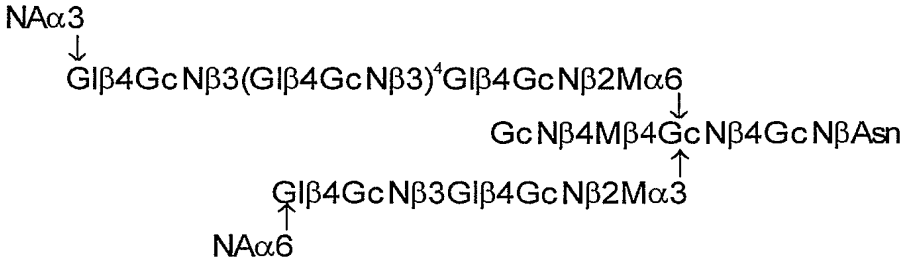
Tamm-Horsfall glycoprotein



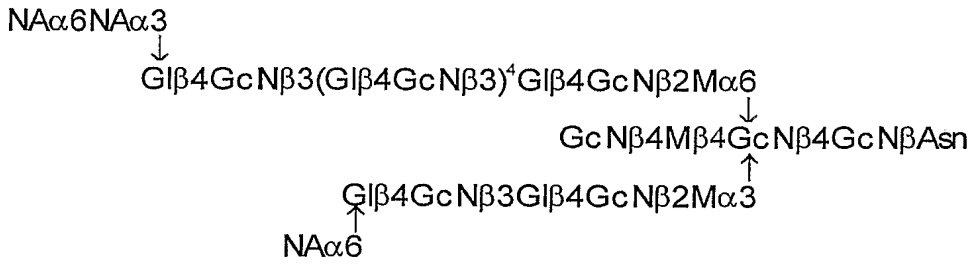
High-mannose type



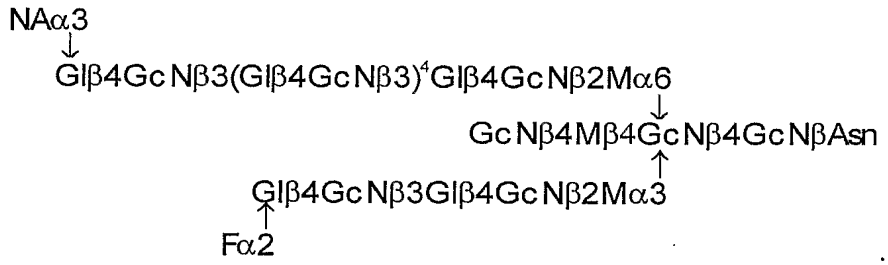
Disialyl foetal erythrocyte antigen



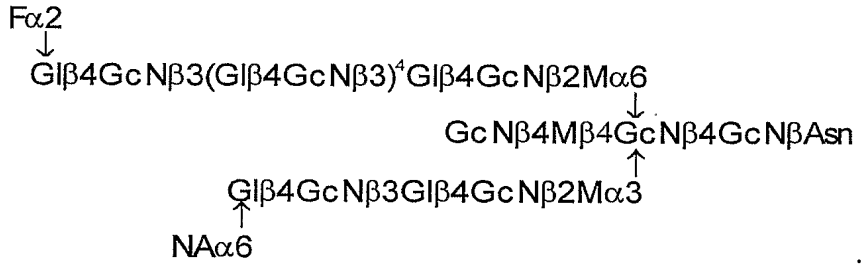
Trisialoyl foetal erythrocyte antigen (disialoyl group on branch)



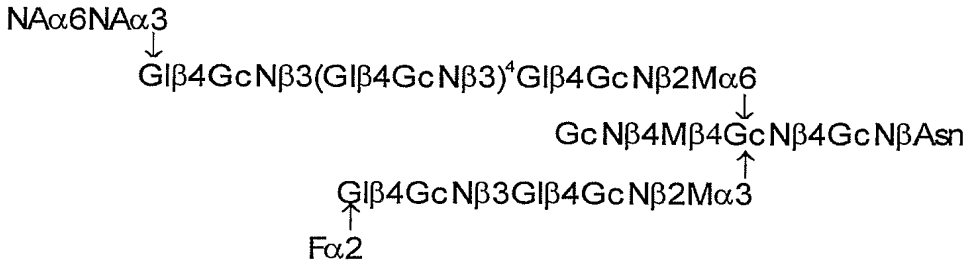
Monofucosyl-monosialyl foetal erythrocyte antigen (fucosylated backbone)



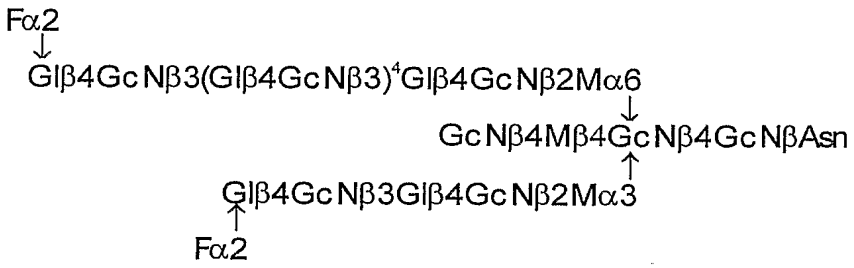
Monofucosyl-monosialyl foetal erythrocyte antigen (fucosylated branch)



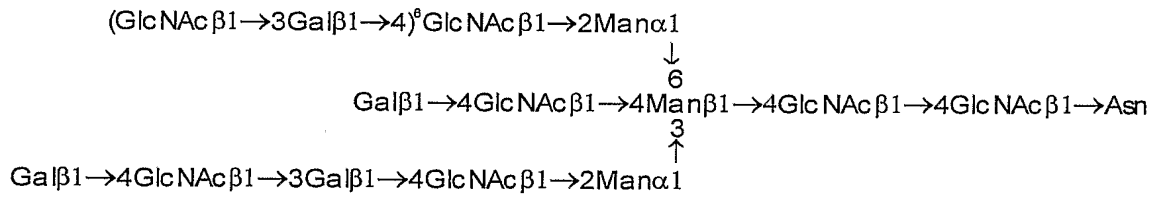
Monofucosyl-disialyl foetal erythrocyte antigen (disialyl group on branch)



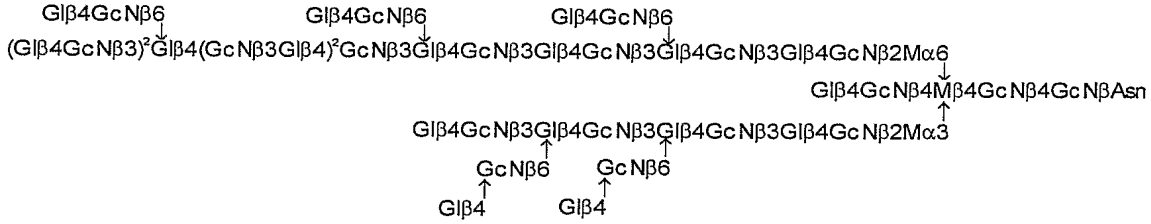
Difucosyl foetal erythrocyte antigen



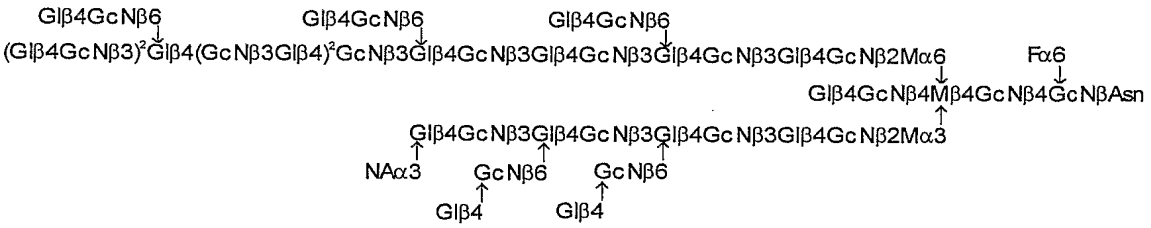
Foetal lactosaminoglycan



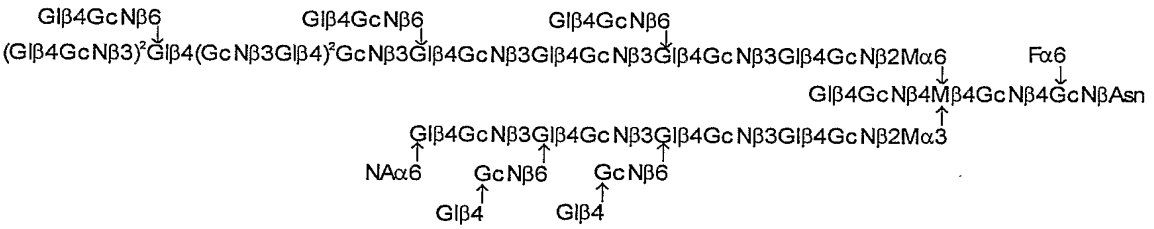
Adult lactosaminoglycan



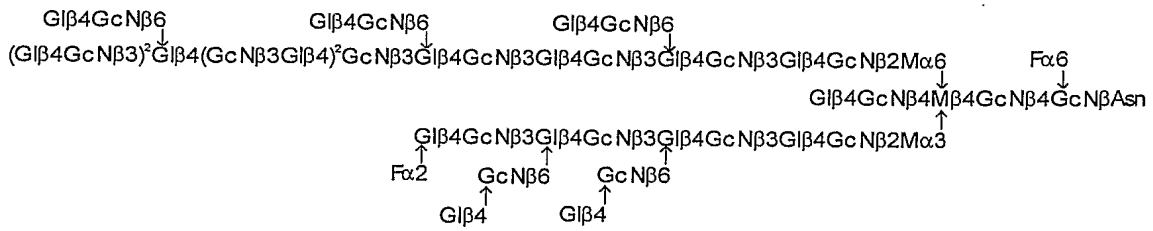
Monofucosyl-monosialoyl adult erythrocyte antigen



Monofucosyl-monosialoyl adult erythrocyte antigen



Difucosyl adult erythrocyte antigen



Key: Gl = D-Gal, Gc = D-Glc, GcN = D-GlcNAc, M = D-Man, F = L-Fuc, NA = NeuAc.

In the context of this description of the invention the term "glycolipid" means a lipid containing carbohydrate of amphipathic character including: glycosylated glycerolipids, such as glycosylated phosphoglycerides and glycosylglycerides; glycosylated sphingolipids (neutral glycolipids) such as glycosylceramides or cerebrosides; and gangliosides (acidic glycolipids).

In the context of this description of the invention the phrase "glycolipid-linked antigen" means a lipid containing carbohydrate in which an antigen (typically a protein) is linked to the glycolipid via the carbohydrate portion of the molecule. Examples of glycolipid-linked antigens include GPI-linked proteins.

It will be understood by those skilled in the art that a glycolipid is itself an antigen. The term and phrase "glycolipid" and "glycolipid-linked antigen" are used to distinguish between naturally occurring molecules where the antigen is the glycolipid and naturally occurring molecules where the antigen is linked to the glycolipid via the carbohydrate portion of the glycolipid. By analogy the "synthetic glycolipids" of the invention could be described as both synthetic membrane anchors and synthetic molecule constructs to the extent that the antigen may be the synthetic glycolipid *per se* or attached to the synthetic glycolipid.

In the context of this description of the invention the term "glycotope" is used to refer to the antigenic determinant located on the carbohydrate portion of a glycolipid. The classification of glycolipid antigens in blood group serology is based on the structure of the carbohydrate portion of the glycolipid.

In blood group serology it is known that the terminal sugars of the glycotopes of A-antigens are GalNAcα1-3(Fucα1-2)Galß, and the terminal sugars of the glycotopes of the B-antigens are Galα1-3(Fucα1-2)Galß. Incorporation into the membrane of RBCs of water soluble synthetic molecule constructs of the invention where F is GalNAcα1-3(Fucα1-2)Galß or Galα1-3(Fucα1-2)Galß provides RBCs that are serologically equivalent to A-antigen or B-antigen expressing RBCs, respectively.

The terminal three sugars of the carbohydrate portion of the naturally occurring A- or B-antigen are the determinant of the A and B blood groupings. The terminal four or five sugars of the

carbohydrate portion of the naturally occurring A-antigen are the determinant of the A blood sub-groupings A type 1, A type 2, etc. Accordingly the RBCs incorporating the synthetic molecule constructs of the invention can be used to characterise and discriminate between blood typing reagents (antibodies) of differing specificity.

It will be understood by those skilled in the art that the carbohydrate portion of a glycolipid may be modified and linked to other antigens by the methods described in the specification accompanying the international application no. PCT/NZ2003/00059 (published as WO03087346).

Water soluble synthetic molecule constructs of the invention that exclude a carbohydrate portion are contemplated by the inventors. Antigens other than carbohydrates or oligosaccharides, but with similar physico-chemical properties, may be substituted for F in the "synthetic glycolipids" described.

Alternative structures of S_1-S_2 for a water soluble synthetic molecule construct (F-S_1-S_2-L) where F is an antigen with similar physico-chemical properties to the carbohydrate portion of the A-, B- or H-antigens of the ABO blood groups and L is a glycerophospholipid.

S_1 is selected from:	S_2 is selected from:
$-O(CH_2)_3NH-$ (2-aminoethyl),	$-CO(CH_2)_2CO-$,
$-O(CH_2)_3NH-$ (3-aminopropyl),	$-CO(CH_2)_3CO-$,
$-O(CH_2)_4NH-$ (4-aminobutyl),	$-CO(CH_2)_4CO-$ (adipate), and
$-O(CH_2)_5NH-$ (5-aminopentyl), and	$-CO(CH_2)_5CO-$
$-O(CH_2)_nNHCO(CH_2)_m-$ n, m = 2 to 5	

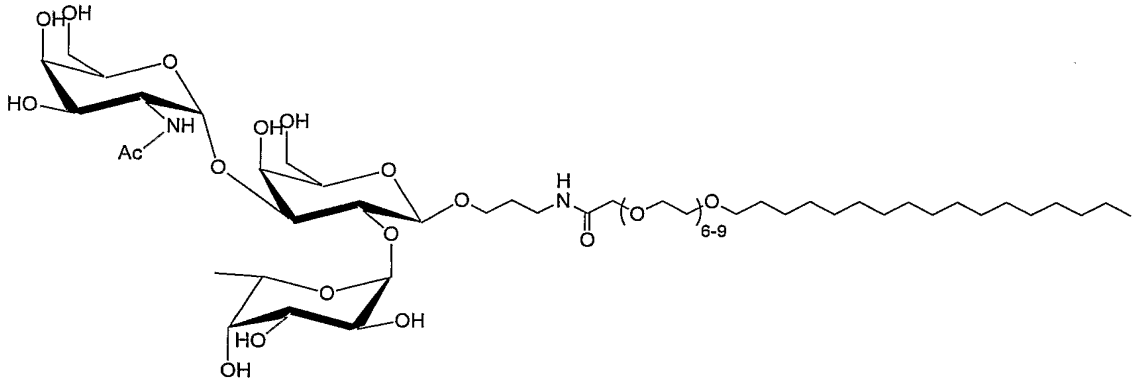
Synthetic molecule constructs of the invention that comprise an antigen (F) with differing physico-chemical properties to those of carbohydrates or oligosaccharides are also contemplated by the inventors. Water soluble synthetic molecule constructs comprising these antigens may be prepared by selecting different spacers.

The advantages provided by the synthetic molecule constructs of this invention will accrue

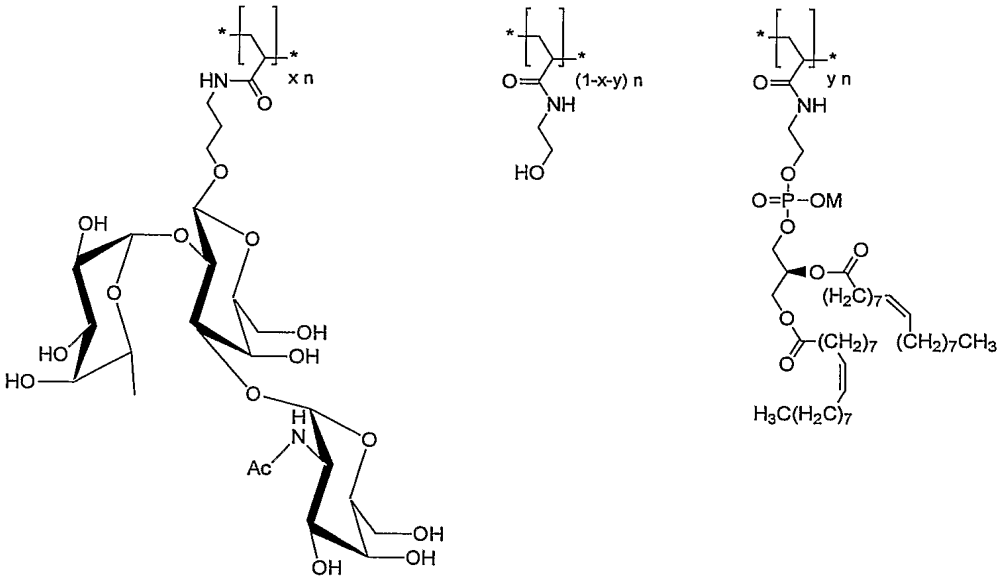
when used in the practice of the inventions described in the specifications for the international application nos. PCT/N02/00212 (published as WO03/034074) and PCT/NZ03/00059 (published as WO03087346). The specifications accompanying these applications are incorporated herein by reference.

A particular advantage is the ability of the synthetic molecule constructs to be used in the transformation of cells at reduced temperatures, e.g. 4°C.

As described herein not all structures of the spacer (S_1-S_2) will provide a synthetic molecule construct (F-S_1-S_2-L) that is water soluble and spontaneously and stably incorporate in to a lipid bilayer such as a cell membrane. In addition to the synthetic molecule constructs of the invention the inventors have prepared synthetic molecule constructs A_{tri}-sp-lipid (**IV**) and Atri-PAA-DOPE (**V**) that were determined not to be water soluble.



A_{tri}-sp-lipid (**IV**)



A_{tri}-PAA-DOPE (**V**) where x, y = 0.05 to 0.2

The invention will now be illustrated by reference to the following non-limiting Examples and Figures of the accompanying drawings in which:

Figure 1 shows Diamed results of Cellstab stored cells transformed by A glycolipid transformation solution at (L to R) 10 mg/mL, 5 mg/mL, 2 mg/mL, 2 mg/mL* and 1 mg/mL. Antisera used are Albaclone (top) and Bioclone (bottom). (* - transformation solution (containing glycolipids) was not washed out after the incubation, it was left in over night and washed out the next day (day 2).)

Figure 2 shows Diamed results of Cellstab stored cells transformed by B glycolipid transformation solution at (L to R) 10 mg/mL, 5 mg/mL, 2 mg/mL, 2 mg/mL* and 1 mg/mL. Antisera used are Albaclone (top) and Bioclone (bottom). (* - transformation solution (containing glycolipids) was not washed out after the incubation, it was left in over night and washed out the next day (day 2)).

Figure 3 shows Diamed results of cells transformed at 4°C by synthetic A glycolipid transformation solution at (L to R): washed 0.08 mg/mL; unwashed 0.08 mg/mL; washed 0.05 mg/mL; unwashed 0.05 mg/mL; washed 0.03 mg/mL; and unwashed 0.03 mg/mL. The antisera used was Bioclone anti-A.

Figure 4 shows cells that were no longer washed prior to testing. Diamed results of cells transformed at 4°C by synthetic A glycolipid transformation solution at (L to R): 0.08 mg/mL, 0.05 mg/mL and 0.03 mg/mL. The antisera used was Bioclone anti-A.

Figure 5 shows in the left column Diamed results of cells transformed at 4°C by synthetic B glycolipid transformation solution at (L to R): washed 0.6 mg/mL; unwashed 0.6 mg/mL; washed 0.3 mg/mL; unwashed 0.3 mg/mL; washed 0.15 mg/mL; and unwashed 0.15 mg/mL; and in the right column Diamed results of cells transformed at 4°C by synthetic B glycolipid transformation solution at (L to R): washed 0.08 mg/mL; unwashed 0.08 mg/mL; washed 0.05 mg/mL; unwashed 0.05 mg/mL; washed 0.03 mg/mL; and unwashed 0.03 mg/mL. The antisera used was Bioclone anti-A.

Figure 6 shows cells that were no longer washed prior to testing. Diamed results of cells transformed at 4°C by synthetic B glycolipid transformation solution at (L to R): 0.6 mg/mL, 0.3 mg/mL and 0.15 mg/mL..

Figure 7 shows Diamed results of cells transformed at 4°C by parallel transformation with synthetic A and B glycolipids. Wells 1 and 2 (L to R) contain washed A 0.07 + B 0.3 mg/mL against anti-A and anti-B. Wells 3 and 4 contain unwashed A 0.07 + B 0.3 mg/mL against anti-A and anti-B.

Figure 8 shows cells that were no longer washed prior to testing. Diamed results of cells transformed at 4°C by parallel transformation with synthetic A and B glycolipids. Wells 1 and 2 (L to R) contain unwashed A 0.07 + B 0.3 mg/mL against anti-A and anti-B.

Figure 9 shows Diamed results of cells transformed at 4°C by parallel transformation with synthetic A and B glycolipids. Wells 1 and 2 (L to R) contain washed A 0.07 + B 0.2 mg/mL against anti-A and anti-B. Wells 3 and 4 contain unwashed A 0.07 + B 0.2 mg/mL against anti-A and anti-B.

Figure 10 shows cells that were no longer washed prior to testing. Diamed results of cells transformed at 4°C by parallel transformation with synthetic A and B glycolipids. Wells 1 and 2 (L to R) contain unwashed A 0.07 + B 0.2 mg/mL against anti-A and anti-B.

Figure 11 shows Diamed results of cells transformed at 4°C by parallel transformation with synthetic A and B glycolipids. Wells 1 and 2 (L to R) contain washed A 0.06 + B 0.3 mg/mL against anti-A and anti-B. Wells 3 and 4 contain unwashed A 0.06 + B 0.3 mg/mL against anti-A and anti-B.

Figure 12 shows cells that were no longer washed prior to testing. Diamed results of cells transformed at 4°C by parallel transformation with synthetic A and B glycolipids. Wells 1 and 2 (L to R) contain unwashed A 0.06 + B 0.3 mg/mL against anti-A and anti-B.

Figure 13 shows Diamed results of cells transformed at 4°C by parallel transformation with synthetic A and B glycolipids. Wells 1 and 2 (L to R) contain washed A 0.06 + B 0.2 mg/mL against anti-A and anti-B. Wells 3 and 4 contain unwashed A 0.06 + B 0.2 mg/mL against anti-A and anti-B.

Figure 14 shows cells that were no longer washed prior to testing. Diamed results of cells transformed at 4°C by parallel transformation with synthetic A and B glycolipids. Wells 1 and 2 (L to R) contain unwashed A 0.06 + B 0.2 mg/mL against anti-A and anti-B.

Figure 15 shows Diamed results of cells transformed at 4°C by parallel transformation with synthetic A and B glycolipids. Wells 1 and 2 (L to R) contain washed A 0.05 + B 0.3 mg/mL against anti-A and anti-B. Wells 3 and 4 contain unwashed A 0.05 + B 0.3 mg/mL against anti-A and anti-B.

Figure 16 shows cells that were no longer washed prior to testing. Diamed results of cells transformed at 4°C by parallel transformation with synthetic A and B glycolipids. Wells 1 and 2 (L to R) contain unwashed A 0.05 + B 0.3 mg/mL against anti-A and anti-B.

Figure 17 shows Diamed results of cells transformed at 4°C by parallel transformation with synthetic A and B glycolipids. Wells 1 and 2 (L to R) contain washed A 0.05 + B 0.2 mg/mL against anti-A and anti-B. Wells 3 and 4 contain unwashed A 0.05 + B 0.2 mg/mL against anti-A and anti-B.

Figure 18 shows cells that were no longer washed prior to testing. Diamed results of cells transformed at 4°C by parallel transformation with synthetic A and B glycolipids. Wells 1 and 2 (L to R) contain unwashed A 0.05 + B 0.2 mg/mL against anti-A and anti-B.

COMPARATIVE EXAMPLES

Comparative Example 1 – Preparation of Natural Glycolipids

Purification by HPLC

In the first stage, columns were packed with dry silica (15-25 μm) before each run. This meant that relatively dirty sample could be used in HPLC because the silica could be discarded along with the theoretically high level of irreversibly bound contaminants.

Glycolipids can be separated on silica gel with a mobile phase of increasing polarity. The program was a linear gradient beginning with 100% chloroform-methanol-water 80:20:1 (v/v) and ending with 100% chloroform-methanol-water 40:40:12 (v/v).

The HPLC equipment used for was a Shimadzu system capable of pumping and mixing four separate solvents at programmed ratios. As chloroform, methanol and water evaporate at different rates, it was decided to develop a program whereby the solvent components were not mixed prior to entering the HPLC.

The Shimadzu HPLC could mix four different liquids by taking a 'shot' from each of four bottles in turn. It was believed that 'shots' of chloroform and water directly next to each other in the lines would cause miscibility problems, so methanol was sandwiched in between these two immiscible components. Additionally, the water was pre-mixed with methanol in a 1:1 ratio to further prevent problems with miscibility. The four pumps were assigned as follows: A – chloroform; B and D – methanol; and C - methanol-water 1:1 (v/v).

Comparative Example 2 – Red Blood Cell Transformation with Natural Glycolipids

Cell agglutination was assessed using the Diamed-ID Micro Typing System in addition to using conventional tube serology. The cards used were NaCl, Enzyme test and cold agglutinin cards, which were not pre-loaded with any antisera or other reagents. This allowed the use of specific antisera with both methodologies.

Table 1 Gel-cards.

Manufacturer	Catalogue ref
Diamed	NaCl, Enzyme test and cold agglutinin cards

A comparative trial was carried out between tube serology and the Diamed system to establish the performance of the two systems. Cells were transformed at 25°C for 4 hours. Seraclone and Alba-clone anti-A sera were used to gauge equivalency. The results are shown in Table 3 below.

Table 2. Antisera used in comparison of Diamed column technology and manual tube serology.

Manufacturer	Catalogue ref	Lot	Expiry
Albaclone, SNBTS	Anti-A.	Z0010770	12.12.04
Seraclone, Biotest	801320100	1310401	12.04.03

Table 3. Agglutination results comparing tube serology with the Diamed column system. Cells were tested against two different anti-A sera – Albaclone and Seraclone.

		A glycolipid (mg/mL)				
		10	5	2	1	0
Tube	Albaclone	3+	2+	0	0	0
	Seraclone	3+	2+	0	0	0
Diamed	Albaclone	2+	2+	0	0	0
	Seraclone	3+	2+	1+	w+	0

In this experiment, the Diamed column platform proved to be more sensitive to the weaker reactions than manual serology with the Seraclone anti-A, but not with Albaclone. These reagents are formulated differently, and are thus not expected to perform identically. However, the fact that Seraclone anti-A manual serology combination did not detect positivity is probably due to operator interpretation. The weaker reactions are notoriously difficult to accurately score, and the difference between 1+ and 0 can be difficult to discern in tubes.

Table 4. Tube serology agglutination of natural glycolipid A transformed cells over different experiments.

Expt	A								
	10	5	2	1	0.1	0.01	0.001	0.0001	0
1	3+	2+	0	0					0
2	4+	3+	2+	1+	w+	0	0	0	0

1 Seraclone (37°C for 1.5 hours Protocol 5.1, variation 1) – see Table 6.

2 Seraclone (25°C for 4 hours Protocol 5.1, variation 3)

Method Development

The variables of glycolipid concentration, incubation temperature, incubation duration, diluent and size of the carbohydrate were examined for their effect on cell health and transformation as assessed by agglutination with the relevant antibody.

Glycolipid Concentration

Initial transformation experiments were carried out with a highly purified (HPLC) Leb sample and a less pure blood group A sample. Transformation was performed at 37°C for 1.5 hours

The A sample contained other lipid impurities, and thus comparatively less blood group A molecules by weight than the Le^b sample of equivalent concentration (w/v). This seems to be borne out by the fact that higher concentrations of the A glycolipid than the Le^b glycolipid were required to produce equivalent agglutination scores (see Table 6).

The level of impurity in the A glycolipid sample may also have contributed to the lower stability over the 62 day period – the A-transformed cells 'died' at the highest concentration (having received the largest dose of impurity).

Table 5 Anti-A and anti-Le^b used in initial testing of natural glycolipid transformation.

Manufacturer	Catalogue ref	Batch number	Expiry
Anti-A			
Seraclone, Biotest	801320100	1310401	12.04.03
Anti-Le^b			
CSL		12801	

Table 6 Stability of RBCs transformed with natural A and Le^b glycolipid as assessed by tube serology agglutination over the period of 62 days.

Glycolipid		Le^b			A		
(mg/mL)	E	Day 1	Day 25	Day 62	Day 1	Day 25	Day 62
10		4+		2-3+	3+	2+	?
5		4+		2-3+	2+	2+	w+
2		3+		1-2+	0	1+	0
1		4+		2+	0	1+	0
0.1		3+	2+	0	0		
0.01		2+	2+	0	0		
0.001		2+	2+	0	0		
0.0001		2+	0	0	0		
0		0	0	0	0	0	0

? – cells unreadable.

1	Seraclone anti-A	CSL anti-Leb
2	-	CSL anti-Leb
3	Seraclone anti-A	CSL anti-Leb
4	Seraclone anti-A	CSL anti-Leb

The above cells were also rated for haemolysis and these results are shown in Table 7 below.

Table 7. Haemolysis as assessed visually. Day 1 – in the supernatant of the first wash after transformation, days 25 and 62 – in the cell preservative solution before the cells are resuspended after storage. Scoring scale is analogous to the 4+ to 0 agglutination scale: hhhh – severely haemolysed, hhh – very haemolysed, hh – moderately haemolysed, h – mildly haemolysed, w – faintly haemolysed and 0 – no haemolysis seen.

Glycolipid concentration (mg/mL)	Haemolysis Leb			A		
	Day 1	Day 25	Day 62	Day 1	Day 25	Day 62
10	h	0	h	h	h	dead
5	hh	0	hhh	w	0	hh
2	w	0	hhh	w	0	hhhhh
1	w	0	hhh	h	0	hhhh
0.1			h			hhh
0.01			hh			
0.001			h			
0.0001			h			
Control	h	0	h	h	h	

These results show that cell haemolysis can be shown to be associated with transformation with high concentrations of glycolipid. It is unclear whether the mechanism underlying this is disruption of the plasma membrane by large amounts of glycolipid being inserted or the rate of that insertion, or is possibly due to the quantity of associated impurity. However, the results for Le^b at day 62 seem to support the first explanation.

The Le^b sample was highly purified – in fact, before being dissolved, it was a powder of pure white colour (this indicates a high level of purity especially when the sample is of erythrocyte origin, as many of the contaminants are pigmented), and thus it is unlikely that the haemolysis was due to the deleterious effect of impurities. It is clear to see that at 62 days, the amount of haemolysis occurring diminishes in line with the decrease in the glycolipid concentration.

Temperature

Experiments were carried out to investigate other possible mechanisms for the reduction of haemolysis of RBCs during the insertion step. Previous experiments had shown that haemolysis was worse at higher glycolipid concentrations than at lower concentrations, and it is thought that haemolysis may also be related to the rate of glycolipid insertion. Since temperature is believed to affect the rate of insertion, experiments were conducted comparing 37°C with RT (25°C), and since the rate was expected to slow down as temperature decreased, the incubation period for the RT experiment was 4 hrs.

Haemolysis was assessed visually and scored following insertion (see Table 8). Serology tests were performed on the cells and the results are shown in Table 8.

Table 8. The effect of incubation temperature on haemolysis and agglutination during insertion of glycolipids into RBC membranes. Haemolysis was scored visually at each of the three washes.

Glycolipid (mg/mL)	Haemolysis						Serology	
	RT			37 °C			RT	37 °C
	wash 1	wash 2	wash 3	wash 1	wash 2	wash 3		
10	w	0	0	hh	w	0	2+	2+
1	w	0	0	hh	h	vw	1+	w+

The effect of incubation temperature on the health of cells as evidenced by haemolysis was negligible.

Duration of Incubation

Incubation at 37°C was carried out for 1 and 2 hours and its effect on cell health and transformation assessed by agglutination with the relevant antibody.

Table 9. Antisera used in the duration of incubation trial.

Manufacturer	Catalogue ref	Batch number	Expiry date
Albaclone, SNBTS	Anti-A.	Z0010770	12.12.04
Bioclone, OCD	Anti-A, experimental reagent	DEV01102	-
Albaclone, SNBTS	Anti-B	Z0110670	01.07.05
Bioclone, OCD	Anti-B, experimental reagent	DEV01103	-

Table 10. Effect of incubation time on agglutination of cells transformed with natural glycolipids.

Glycolipid	Concentration (mg/mL)	Albaclone		BioClone	
		1 hour	2 hours	1 hour	2 hours
A	10	4+	4+	4+	4+
	5	4+	4+	4+	2+
	2	4+	3+	3+	2+
	1	3+	2+	2+	2+
	0.5	2+	2+	1+	w+
B	10	3+	2+	4+	1+
	5	3+	2+	3+	2+
	2	2+	2+	2+	1+
	1	1+	w+	1+	w+
	0.5	1+	w+	w+	w+

These results indicate that increasing the duration of incubation during natural glycolipid insertion does not enhance transformation. In fact, the agglutination scores are reduced after the two hour incubation. This may be due to the destabilisation of the membrane or exchange of the glycolipids back into solution.

Transformation Solution Diluent

Experiments were also carried out to determine if changing the glycolipid diluent solution could reduce haemolysis. Working strength PBS was compared with 2 x PBS and 2 % Bovine Serum Albumin (BSA) in working strength PBS. Cells were incubated at 37°C for 1.5 hours. The results are shown in Table 11 below.

Table 11. Study on the effect on haemolysis of changing the glycolipid diluent solutions during insertion of glycolipids into RBC membranes.

Glycolipid concentration (mg/mL)	Glycolipid Diluent Solution		
	PBS	2 x PBS	2% BSA in PBS
40	hhh	hhh	hhh
30	hhh	hhh	hhh
20	hhh	hhh	hhh
10	hhh	hhh	hhh
0	0	0	0

Stability Trials

Transformation at 25°C.

Table 12. Early stability trial of cells transformed with natural A glycolipid.

Expt	Day	A								
		10	5	2	1	0.1	0.01	0.001	0.0001	0
1	7	4+	3-4+	1+	0	0	0	0	0	0
2	43	3+	w+	0	0	0	0	0	0	0
3	50	1+	0	0	0					
4	60	3+	1+	0						
5	67	w+	vw	vw						
6	74	2+	0	0						
7	81	2+	1+	0						

1	Seraclone
2	Alba
3	Alba
4	Alba
5	Alba
6	Alba
7	Alba

Once A and B blood group glycolipids had been HPLC purified to an acceptable level, an experiment to find the appropriate concentrations for stability trials was carried out.

Table 13. Antisera used in stability trials (Table 14 and Table 15).

Manufacturer	Catalogue ref	Batch number	Expiry date
Albaclone, SNBTS	Anti-A.	Z0010770	12.12.04
Bioclone, OCD	Anti-A, experimental reagent	DEV01102	-
Albaclone, SNBTS	Anti-B	Z0110670	01.07.05
Bioclone, OCD	Anti-B, experimental reagent	DEV01103	-

Table 14. Tube serology of O RBCS transformed with A glycolipid in order to establish appropriate concentrations for stability trials.

Anti-A	Expt	A glycolipid (mg/mL)								
		10	5	2	1	0.5	0.1	0.01	0.001	0
Alba	1	3+	2+	1+	0		0	0	0	0
	2	4+	4+	3+	2+	w+				
Bio	1	3+	2+	1+	0		0	0	0	0
	2	4+	4+	3+	2+	w+				

1 & 2 25°C for 4 hours

Table 15. Tube serology of O RBCS transformed with B glycolipid in order to establish appropriate concentrations for stability trials.

Anti-B	Expt	B glycolipid (mg/mL)								
		10	5	2	1	0.5	0.1	0.01	0.001	0
Alba	1	2+	1+	w+	0		0	0	0	0
	2	1+	1+	w+	0	w+				
Bio	1	3+	2+	w+	0		0	0	0	0
	2	1+	1+	w+	0	w+				

1 & 2 25°C for 4 hours

From these two trials, it was decided to transform cells for stability trialling using concentrations of 5, 2, 1 and 0.1 mg/mL A and B blood group glycolipids.

Two sets of cells were transformed with different concentrations of A glycolipid. Transformation was performed at 25°C. One set was tested at weeks 1 and 6, and the other was tested weekly for agglutination. The agglutination results from tube serology and Diamed are shown in Table 16 below. All cells were stored in Cellstab, in bottles with flat bases. The cells showed minimal to no haemolysis at any time. The reagents used in the stability trial are shown in Table 13.

Table 16. Agglutination results for cells transformed with different concentrations of A glycolipid. Results were obtained using Albaclone anti-A.

		A glycolipid (mg/mL)					
		10	5	2	1	0.1	control
Long term testing							
Day 1	Tube	4+	3+	2+	1+	+w	0
	Diamed	3+	3+	+w	0	0	0
Day 17	Tube	3+	2+	0	0		0
	Diamed	3+	2+	1+	0		0
Weekly testing							
Day 1	Tube	3+		2+		0	0
	Diamed	3+		0		0	0
Day 8	Tube	1+		0		0	0
	Diamed	3+		0		0	0
Day 15	Tube	1+		0		0	0
	Diamed	3+		2+		0	0
Day 22	Tube	3+		0		0	0
	Diamed	3+		0		0	0
Day 29	Tube	*+w		*0		*0	*0
	Diamed	*3+		*0		*0	*0
Day 36	Tube	*		*		*	*0
	Diamed	*3+		*0		*0	*0
Day 43	Tube	*		*		*	*0
	Diamed	*		*		*	*0

* - Albaclone, while all others used Seraclone anti-A.

Comparison of the two cell storage solutions, Celpresol (CSL) and Cellstab (Diamed) was carried out to test their relative abilities to support modified RBCs. Cells were transformed at 25°C for 4 hours.

Table 17. Comparison of cell storage solutions on agglutination of natural glycolipid transformed cells.

Soln	A-PAA	Testing day											
	(mg/mL)	2	8	15	22	28	36	43	51	59	66	73	80
CS	10	3+	1+	1+	3+	3+	2+	3+	1+	2+	2+	vw	2+
	2	2+	0	0	0	0	0	0					
	0.1	0	0	0	0	0	0	0					
CP	10		2+	2+	3+	2+	1+	2+	2+	0	w+	1+	1+
	2		0	0	0	0	vw	0					
	0.1		0	0	0	0	0	0					

CS – Cellstab

CP – Celpresol

2	Seraclone
8	Seraclone
15	Seraclone
22	Alba
28	Alba
36	Alba
43	Alba
51	Alba
59	Alba
66	Alba
73	Alba
80	Alba

B glycolipids were used to transform cells at 25°C for four hours.

Table 18. B glycolipids.

		B glycolipid (mg/mL)		
		20	10	5
1	Alba	2+	1+	0
8	Bio	vw	0	0

1 Albaclone anti-B
2 Bio

The results below (Table 19) are derived from cells that were transformed at 25°C for 4 and 6 hours. There appears to be some strengthening of the agglutination reaction upon the addition of 2 hours to the incubation. However, this difference does not appear to be significant after the first week.

Table 19. B glycolipids.

Day	Transformation time	Antisera	B glycolipid (mg/mL) 40	30	20	10
1	4 hours	Alba	2+	2+	1-2+	1+
		Bio	1+	2-3+	1-2+	1+
	6 hours	Alba			?	?
		Bio			?	?
8	4 hours	Alba	0	0	0	vw
		Bio	vw	vw	0	1+
	6 hours	Alba			1+	0
		Bio			2+	vw
11	4 hours	Alba	0	0	0	0
		Bio	w+	w+	vw	0
	6 hours	Alba			0	0
		Bio			1+	0
18	4 hours	Bio	w+	w+	1+	1+
	6 hours	Bio			vw	vw
24	4 hours	Alba	vw	vw	vw	vw
		Bio	w+	w+	1+	w+
	6 hours	Alba			vw	vw
		Bio			w+	w+

1	Alba and Bio
8	Alba and Bio
11	Alba and Bio
18	Bio
24	Alba and Bio

Stability Trial

The purpose of this trial is to establish the stability of RBCs transformed with blood group A and B antigen solutions of varying concentrations when stored in two different cell preservative solutions – Cellstab and Alsevers.

A and B antisera from two different sources were used in serology testing.

All cells were tested using the standard tube serology platform up to 42 days, at which time the cell agglutination reactions had become too difficult to score manually (see Table 20 for A results and Table 21 for B results).

Diamed gel-card testing was carried out to day 56 for the Alsevers stored cells, and discontinued at day 63 due to fungal contamination (although still returning positive scores).

The Cellstab stored cells continued to be tested up to day 70, and were still viable at this point (see Figure 1 for A results and Figure 2 for B results).

The reagents used in the stability trial are shown in Table 13.

Table 20. Tube serology results of stability trial of cells transformed with varying concentrations of A glycolipid and stored in either Cellstab or Alsevers.

Day	Cell storage solution	Albaclone Anti-A (SNBTS) Transformation Solution (mg/mL)					Bioclone Anti-A (OCD – Developmental reagent)				
		10	5	2	2*	1	10	5	2	2*	1
2	Alsevers	4+	3+	2+	1+	w+	3+	3+	1+	1+	0
	Cellstab	4+	4+	3+	1+	1+	3+	3+	2+	1+	0
8	Alsevers	4+	4+	2+	1+	1+	2+	2+	1+	1+	0
	Cellstab	4+	4+	3+	2+	1+	3+	3+	2+	w+	0
14	Alsevers	4+	3+	2+	2+	w+	2+	1+	w+	vw	0
	Cellstab	4+	3+	3+	2+	w+	3+	2+	w+	vw	0
21	Alsevers	3+	2+	2+	2+	1+	2+	2+	2+	1+	0
	Cellstab	3+	3+	2+	+	‡	2+	‡	‡	‡	0
28	Alsevers	2+	2+	1+	1+	0	2+	2+	1+	1+	0
	Cellstab	2+‡	2+‡	‡	‡	0	1+	w+	0	0	0
36	Alsevers	3+	2+	2+	2+	1+	3+	3+	2+	1+	1+
	Cellstab	3+‡	2+‡	‡	‡	‡	3+‡	‡	‡	‡	‡
42	Alsevers	3+	3+	1+	w+	0	2+	2+	2+	1+	1+
	Cellstab	4+‡	4+‡	‡	‡	‡	‡	‡	‡	‡	0

* - transformation solution (containing glycolipids) was not washed out after the incubation, it was left in over night and washed out the next day.

‡ - positive cell button, but cells fall off as negative (score assignment impossible).

Table 21. Tube serology results of stability trial of cells transformed with varying concentrations of B glycolipid and stored in either Cellstab or Alsevers.

Day	Cell storage solution	Albaclone Anti-B (SNBTS) Transformation Solution (mg/mL)					Bioclone Anti-B (OCD - Developmental reagent)				
		10	5	2	2*	1	10	5	2	2*	1
2	Alsevers	3+	3+	1+	1+	1+	2+	1+	1+	1+	0
	Cellstab	3+	3+	2+	2+	1+	2+	2+	2+	1+	w+
8	Alsevers	1+	1+	w+	0	0	0	0	0	0	0
	Cellstab	2+	1+		w+	0	1+	1+	w+	0	0
14	Alsevers	2+	2+	0	w+	0	0	1+	1+	2+	0
	Cellstab	1+	w+	0	0	0	2+	2+	w+	1+	1+
21	Alsevers	‡	‡	‡	‡	‡	1	1	‡	‡	‡
	Cellstab	‡	‡	‡	‡	‡	+	+	+	‡	‡
28	Alsevers	2+	1+	w+	0	0	2+	1+	2+	0	0
	Cellstab	‡	‡	‡	0	0	‡	0	‡	‡	0
36	Alsevers	2+	2+	2+	1+	1+	2+	2+	2+	1+	1+
	Cellstab	‡	‡	‡	‡	‡	‡	‡	‡	‡	‡
42	Alsevers	2+	2+	2+	2+	w+	2+	2+	1+	w+	w+
	Cellstab	‡	‡	‡	‡	‡	‡	‡	‡	‡	‡

* - transformation solution (containing glycolipids) was not washed out after the incubation, it was left in over night and washed out the next day.

‡ - positive cell button, but cells fall off as negative (score assignment impossible).

EXAMPLES

Example 1 - Preparation of synthetic molecule constructs ("synthetic glycolipids")

Materials and methods

TLC analysis was performed on silica gel 60 F_{254} plates (Merck), the compounds were detected by staining with 8% of phosphoric acid in water followed by heating at over 200°C. Column chromatography was carried out on silica gel 60 (0.2-0.063mm, Merck) or Sephadex LH-20 (Amersham). ^{1}H NMR spectra were acquired on Bruker DRX-500 spectrometer, chemical shifts are given in ppm (δ) relative to CD_3OD.

Synthesis of activated 1,2-O-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE) and 1,2-O-distereoyl-sn-glycero-3-phosphatidylethanolamine (DSPE)(glycerophospholipids)

To a solution of *bis*(N-hydroxysuccinimidyl) adipate (A) (70 mg, 205 μmol) in dry N,N-dimethylformamide (1.5 ml) were added DOPE or DSPE(L) (40μmol) in chloroform (1.5 ml) followed by triethylamine (7 μl). The mixture was kept for 2 h at room temperature, then neutralized with acetic acid and partially concentrated in *vacuo*.

Column chromatography (Sephadex LH-20, 1:1 chloroform-methanol, 0.2% acetic acid) of the residue yielded the activated lipid (A-L) (37 mg, 95%) as a colorless syrup; TLC (chloroform-methanol-water, 6:3:0.5): Rf = 0.5 (DOPE-A), Rf = 0.55 (DSPE-A).

^{1}H NMR ($CDCl_3/CD_3OD$, 2:1), δ:

DOPE-A - 5.5 (m, 4H, 2×(-CH=CH-), 5.39 (m, 1H, $-OCH_2-CHO-CH_2O-$), 4.58 (dd, 1H, J=3.67, J=11.98, -CCOOHCH-CHO-CH_2O-), 4.34 (dd, 1H, J=6.61, J=11.98, -CCOOHCH-CHO-CH_2O-), 4.26 (m, 2H, PO-$CH_2-CH_2-NH_2$), 4.18 (m, 2H, -CH_2-OP), 3,62 (m, 2H, PO-$CH_2-CH_2-NH_2$), 3.00 (s, 4H, ONSuc), 2.8 (m, 2H, -CH_2-CO (Ad), 2.50 (m, 4H, 2×(-CH_2-CO), 2.42 (m, 2H, -CH_2-CO (Ad), 2.17 (m, 8H, 2×(-CH_2-CH=CH-CH_2-), 1.93 (m, 4H, $COCH_2CH_2CH_2CH_2CO$), 1.78 (m, 4H, 2×($COCH_2CH_2$-), 1,43, 1.47 (2 bs, 40H, 20 CH_2), 1.04 (m, 6H, 2 CH_3).

DSPE-A - 5.39 (m, 1H, $-OCH_2-CHO-CH_2O-$), 4.53 (dd, 1H, J=3.42, J=11.98, -CCOOHCH-CHO-CH_2O-), 4.33 (dd, 1H, J=6.87, J=11.98, -CCOOHCH-CHO-CH_2O-), 4.23 (m, 2H, PO-$CH_2-CH_2-NH_2$), 4.15 (m, 2H, -CH_2-OP), 3,61 (m, 2H, PO-$CH_2-CH_2-NH_2$), 3.00 (s, 4H, ONSuc), 2.81 (m, 2H, -CH_2-CO (Ad), 2.48 (m, 4H, 2×(-CH_2-CO), 2.42 (m, 2H, -CH_2-CO (Ad), 1.93 (m, 4H, $COCH_2CH_2CH_2CH_2CO$), 1.78 (m, 4H, 2×($COCH_2CH_2$-), 1,43, 1.47 (2 bs, 40H, 20 CH_2), 1.04 (m, 6H, 2 CH_3).

Condensing activated DOPE (or DSPE) with aminopropylglycosid.

To a solution of activated DOPE (or DSPE) (33μmol) in N,N-dimethylformamide (1 ml) 30 μmol of Sug-sp(sp_1)NH_2 (F-S_1) and 5μl of triethylamine. were added. (For example, the Sug may be either the aminopropyl glycoside (F-S_1) of either GalNAcα1-3(Fucα1-2)Galß trisaccharide (A-glycotope) or Galα1-3(Fucα1-2)Galß trisaccharide (B-glycotope). The mixture was stirred for 2 h at room temperature.

Columns chromatography (Sephadex LH-20 in 1:1 chloroform-methanol, followed by silica gel in ethyl acetate-isopropanol-water, 4:3:1) of the mixture yielded 85-90% of the synthetic molecule constructs designated I to III and VI to IX (Table 2) (For example, A_{tri}-sp-Ad-DOPE (**I**) or B_{tri}-sp-Ad-DOPE (**VI**)).

^{1}H NMR ($CDCl_3/CD_3OD$, 1:1):

A_{tri}-sp-Ad-DOPE (**I**) - δ 5.5 (m, 4H, 2×(-CH=CH-), 5.43-5,37 (m, 2H, H-1 (GalNHAc) and -$OCH_2-CHO-CH_2O$-), 5.32 (d, 1H, H-1, J=3.5 H-1 Fuc), 2.50 (m, 4H, 2×(-CH_2-CO), 2.40 (m, 4H, $COCH_2CH_2CH_2CH_2CO$), 2.20 (m, 8H, 2×(-CH_2-CH=CH-CH_2-), 2.1 (s, 3H, NHAc), 1.92 (m, 2H,

O-$CH_2C\underline{H}_2CH_2$-NH), 1.8 (m, 8H, $COCH_2C\underline{H}_2C\underline{H}_2CH_2CO$ and 2×($COCH_2C\underline{H}_2$-), 1,43, 1.47 (2 bs, 40H, 20 CH_2), 1.40 (d, 3H, J= 6.6, CH_3 Fuc), 1.05 (m, 6H, 2 CH_3).

A_{tri}-spsp_1-Ad-DOPE (**II**) - δ 5.5 (m, 4H, 2×(-C<u>H</u>=C<u>H</u>-), 5.43-5,37 [m, 2H, H-1 (GalNHAc) and -OCH_2-C<u>H</u>O-CH_2O-], 5.32 (d, 1H, H-1, J=3.6 H-1 Fuc), 2.50 (m, 4H, 2×(-$C\underline{H}_2$-CO), 2.40- 2.32 (m, 6H, $COC\underline{H}_2CH_2CH_2C\underline{H}_2CO$ and $COC\underline{H}_2$- (sp_1), 2.18 [m, 8H, 2×(-$C\underline{H}_2$-CH=CH-$C\underline{H}_2$-)], 2.1 (s, 3H, NHAc), 1.95(m, 2H, O-$CH_2C\underline{H}_2CH_2$-NH), 1.8 [m, 10H, $COCH_2C\underline{H}_2C\underline{H}_2CH_2CO$, 2×($COCH_2C\underline{H}_2$-...), -$COCH_2\underline{CH}_2$ $(CH_2)_3$NH-], 1.68 (m, 2H, $CO(CH_2)_3C\underline{H}_2CH_2$NH-), 1,43, 1.47 (2 bs, 42H, 22 CH_2), 1.37 (d, 3H, J=5.6, CH_3 Fuc), 1.05 (m, 6H, 2 CH_3).

A_{tri}-sp-Ad-DSPE (**III**) - δ, 5.42-5.38 (m, 2H, H-1 (GalNHAc) and -OCH_2-C<u>H</u>O-CH_2O-), 5.31 (d, 1H, H-1, J=3.5 H-1 Fuc), 2.48 [m, 4H, 2×(-$C\underline{H}_2$-CO)], 2.42 (m, 4H, $COC\underline{H}_2CH_2CH_2C\underline{H}_2CO$), 2.18 (s, 3H, NHAc), 1.95 (m, 2H, O-$CH_2C\underline{H}_2CH_2$-NH), 1.8 [m, 8H, $COCH_2C\underline{H}_2C\underline{H}_2CH_2CO$ and 2×($COCH_2C\underline{H}_2$-)], 1,43, 1.47 (2 bs, 56H, 28 CH_2), 1.38 (d, 3H, J=6.6, CH_3 Fuc), 1.05 (m, 6H, 2 CH_3).

B_{tri}-sp-Ad-DOPE (**VI**) δ 5.5 (m, 4H, 2×(-C<u>H</u>=C<u>H</u>-), 5.42-5,38 [m, 2H, H-1 (Gal) and -OCH_2-C<u>H</u>O-CH_2O-], 5.31 (d, 1H, H-1, J=3.7, H-1 Fuc), 2.48 [m, 4H, 2×(-$C\underline{H}_2$-CO)], 2.39 (m, 4H, $COC\underline{H}_2CH_2CH_2C\underline{H}_2CO$), 2.18 [m, 8H, 2×(-$C\underline{H}_2$-CH=CH-$C\underline{H}_2$-)], 1.93 (m, 2H, O-$CH_2C\underline{H}_2CH_2$-NH), 1.8 [m, 8H, $COCH_2C\underline{H}_2C\underline{H}_2CH_2CO$ and 2×($COCH_2C\underline{H}_2$-)], 1,43, 1.47 (2 bs, 40H, 20 CH_2), 1.36 (d, 3H, J=6.6, CH_3 Fuc), 1.05 (m, 6H, 2 CH_3).

H_{tri}-sp-Ad-DOPE (**VII**) δ 5.5 [m, 4H, 2×(-C<u>H</u>=C<u>H</u>-)], 5.4 (m, 1H, -OCH_2-C<u>H</u>O-CH_2O-), 5.35 (d, 1H, H-1, J=3.2, H-1 Fuc), 4.65, 4.54 (2d, J=7.4, J=8.6, H-1 Gal, H-1 GlcNHAc), 4.46 (dd, 1H J=3.18, J=12, -CCOO<u>H</u>CH-CHO-CH_2O-), 4.38-4.28 (m, 2H, H-5 Fuc, CCOOHC<u>H</u>-CHO-CH_2O-), 2.48 [m, 4H, 2×(-$C\underline{H}_2$-CO)], 2.40 (m, 4H, $COC\underline{H}_2CH_2CH_2C\underline{H}_2CO$), 2.18 [m, 8H, 2×(-$C\underline{H}_2$-CH=CH-$C\underline{H}_2$-)], 2.08 (s, 3H,NHAc), 1.92 (m, 2H, O-$CH_2C\underline{H}_2CH_2$-NH), 1.82-1.72 [m, 8H, $COCH_2C\underline{H}_2C\underline{H}_2CH_2CO$ and 2×($COCH_2C\underline{H}_2$-)], 1,48, 1.45 (2 bs, 40H, 20 CH_2), 1.39 (d, 3H, J=6.5, CH_3 Fuc), 1.05 (m, 6H, 2 CH_3).

H_{di}-sp-Ad-DOPE (**VIII**) δ 5.49 (m, 4H, 2×(-C<u>H</u>=C<u>H</u>-), 5.37 (m, 1H, -OCH_2-C<u>H</u>O-CH_2O-), 5.24 (d, 1H, H-1, J=2.95, H-1 Fuc), 4.46 (d, J=7.34, H-1 Gal), 2.48 [m, 4H, 2×(-$C\underline{H}_2$-CO)], 2.42-2.35 (m, 4H, $COC\underline{H}_2CH_2CH_2C\underline{H}_2CO$), 2.17 [m, 8H, 2×(-$C\underline{H}_2$-CH=CH-$C\underline{H}_2$-)], 1.95 (m, 2H, O-$CH_2C\underline{H}_2CH_2$-NH), 1.81-1.74 [m, 8H, $COCH_2C\underline{H}_2C\underline{H}_2CH_2CO$ and 2×($COCH_2C\underline{H}_2$-)], 1,45, 1.41 (2 bs, 40H, 20 CH_2), 1.39 (d, 3H, J=6.5, CH_3 Fuc), 1.03 (m, 6H, 2 CH_3).

Galβ-sp-Ad-DOPE (**IX**) - δ 5.51 [m, 4H, 2×(-C<u>H</u>=C<u>H</u>-)], 5.4 (m, 1H, -OCH_2-C<u>H</u>O-CH_2O-), 4.61 (dd, 1H J=3.18, J=12, -CCOO<u>H</u>CH-CHO-CH_2O-), 4.41 (d, J=7.8, H-1 Gal), 4.37 (dd, 1H, J=6.6, J=12, -CCOOHC<u>H</u>-CHO-CH_2O-), 2.50 [m, 4H, 2×(-$C\underline{H}_2$-CO)], 2.40 (m, 4H, $COC\underline{H}_2CH_2CH_2C\underline{H}_2CO$), 2.20 [m, 8H, 2×(-$C\underline{H}_2$-CH=CH-$C\underline{H}_2$-)], 1.97 (m, 2H, O-$CH_2C\underline{H}_2CH_2$-

NH), 1.82-1.72 [m, 8H, $COCH_2C\underline{H_2}C\underline{H_2}CH_2CO$ and $2\times(COCH_2C\underline{H_2}-)$], 1,48, 1.45 (2 bs, 40H, 20 CH_2), 1.05 (m, 6H, 2 CH_3).

Example 2 – Solubility of synthetic glycolipids

Lipid Anchor Evaluation

The first criterion that synthetic glycolipids must meet is that they are soluble in aqueous solvents e.g. phosphate buffered saline. A number of techniques, including heat and/or sonication, were employed in order to maximise this solubility.

The synthetic glycolipid must also be able to insert into the membrane and be recognisable to the appropriate antibody. Initial tests on the molecules were to establish these factors and thus quickly eliminate those molecules that were unsuitable. The results of these initial tests can be seen in Table 23.

Table 22. The range of synthetic glycolipid molecules tested.

Ad-DOPE Lipid Tails:

B_{tri}-sp-Ad-DOPE (**VI**)

A_{tri}-sp-Ad-DOPE (**I**)

Galβ-sp-Ad-DOPE (**IX**)

H_{di}-sp-Ad-DOPE (**VIII**)

H_{tri}-sp-Ad-DOPE (**VII**)

A_{tri}-spsp1-Ad-DOPE (**II**)

Fluo-Ad-DOPE (**XI**)

Different Lipid Tails:

Lipophilic Atri (**?**)

A_{tri}-sp-lipid (**IV**)

B_{tri}-PAA-PEA (**XII**)

A_{tri}-sp-Ad-DSPE (**III**)

Table 23. Solubility of synthetic glycolipids in hot PBS and transformation ability.

Synthetic	Water solubility	Transformation ability
Lipophilic A_{tri}	Yes§	No
A_{tri}-sp-lipid	No	No
B_{tri}-PAA-PEA	No	No
B_{tri}-sp-Ad-DOPE	Yes	Yes
A_{tri}-sp-Ad-DOPE	Yes	Yes
Galβ-sp-Ad-DOPE	Yes	No*
H_{di}-sp-Ad-DOPE	Yes	No*
H_{tri}-sp-Ad-DOPE	Yes	Yes
A_{tri}-$spsp_1$-Ad-DOPE	Yes	Yes
A_{tri}-sp-Ad-DSPE	Yes	Yes

§ - never tried to dissolve this sample in 70°C PBS, but transferred from CM 2:1 by drying onto salts.

* - lack of transformation ability thought to be due to inability of the antibody to recognise this molecule.

Lipophilic A_{trl} had a single rather than a bilipid tail, and it was proposed that there was no insertion into the membrane bilayer.

Solubility in Chloroform

Sample is 30 mg/mL in de-ionised water. Place 100 µL (3 mg) of sample into a separate pre-weighed tube and place this tube in a quickfit tube to dry down on the rotavapor. Add about 500 µL of chloroform to the dry sample and gently shake to dissolve. Remove about half of the volume (1.5 mg) to another pre-weighed tube and dry down both the tubes on the rotavapor. Weigh the tubes to determine the sample weights.

The two tubes had roughly equal weights of sample in them, indicating that the synthetic molecules are readily soluble in chloroform.

Example 3 – Low temperature transformation of RBCs by A_{trl} and B_{trl} synthetic glycolipids

RBCs are healthier when stored at 4°C, and likewise are believed to be healthier when transformed at 4°C. It was not thought that insertion would occur at 4°C due to previous studies (Schwarzmann, 2000). These data were for natural glycolipids. Surprisingly these studies did not predict the behaviour of the synthetic molecules.

Whilst not wishing to be bound by theory this may be due to the physicochemical properties of the natural glycolipid tail, comprised of a sphingolipid and a fatty acid. The diacyl tail of the synthetic glycolipids possibly retains greater fluidity at these lower temperatures. Alternatively, the domain of the plasma membrane that the diacyl tail of the synthetic glycolipids inserts into may retain this greater fluidity. Either explanation may account for insertion of the exogenous synthetic glycolipids. By contrast, it is known that the sphingolipid tails of natural glycolipids congregate in rigid domains and these domains may not allow further incorporation of glycolipid at low temperatures.

Transformation of RBCs with synthetic glycolipids with different lipid tails was first evaluated (Table 25).

Table 24. Antisera used to obtain results presented in Tables 24 to 29.

Anti-A			
Manufacturer	Catalogue ref	Batch number	Expiry date
Albaclone, SNBTS		Z0010770	12.12.04
BioClone, OCD	Experimental reagent	01102	-
Anti-B			
Manufacturer	Catalogue ref	Batch number	Expiry date
Albaclone, SNBTS		Z0110600	27.04.03
BioClone, OCD	Experimental reagent	01103	-

Table 25. Evaluation of insertion of different lipid tails by agglutination with the relevant antisera.

Molecule	Anti-sera	Transformation solution (µg/mL)										
		1000	500	250	125	100	60	50	40	30	20	10
A-DOPE	Alba							w+	w+	0	0	0
	Bio							2+	1+	w+	0	0
	Alba			4+		3+		2-3+				2+
	Bio			4+*		4+*		3+*				3+
	DBA	0										
B-DOPE	Alba	3+										
	Bio	3+										
	Alba	2+	2+	1+	0		0					
	Bio	3+	2+	1+	0		0					
A-sp2	Alba							0	0	0	0	0
	Bio							0	0	0	0	0
	Alba			4+		3+		2+				2+
	Bio			4+*		3-4+*		3+*				2+
	DBA	0										
A-lipid	Alba	0										
	Bio	0										
A-DSPE	Alba							0	0	0	0	0
	Bio							0	0	0	0	0
	Alba			2-3+		2-3+		2+				2+
	Bio			3+		2-3+		2+				2+
	DBA	0										
B-PEA	Alba	0										
	Bio	0										

* - splatter.

Transformation of RBCs with synthetic glycolipids A_{tri} and B_{tri} at 4°C was then evaluated (Tables 25 to 27). These transformations were directed towards the preparation of weak A, B and AB cells

For weak A and B cells transformation solutions (20 µL, A 0.08, 0.05 and 0.03 mg/mL, and B 0.6, 0.3, 0.15, 0.08, 0.05 and 0.03 mg/mL) in 1 x PBS were mixed with washed, packed group

O RBCs (60 µL).

For weak AB cells transformation solutions (20 µL, A 0.07, 0.06 and 0.05 mg/mL, and B 0.3, and 0.2 mg/mL) in 1 x PBS were combined in block titre with washed, packed group O RBCs (60 µL). The combinations were: A 0.07 + B 0.3, A 0.07 + B 0.2, A 0.06 + B 0.3, A 0.06 + B 0.2, A 0.05 + B 0.3, and A 0.05 + B 0.2 mg/mL.

Cells and transformation solutions were placed in a 4°C fridge. Pipette mixing was performed hourly. Cells were removed for testing at intervals against the relevant antisera, and were tested in both washed and unwashed states (i.e. washed samples had the transformation solution removed).

After 48 hours Celpresol™ was added to the cells so that the final cells:non-cells ratio was 3:5 (v/v). The cells continued to be tested at intervals. Testing was discontinued after 8 days because cells turned brown.

This discolouration could be attributed to a number of factors including: cells were already 21 days old when transformed; 8 hour transformation was in PBS not Celpresol™ so cells stressed for this time; and cells may have been mishandled in transit between the transforming and testing laboratories.

Table 26. Diamed results of weak A RBCs transformed at 4°C against anti-A.

Time	Atri (mg/mL)					
	Washed			unwashed		
	0.08	0.05	0.03	0.08	0.05	0.03
2 hrs	0	0	0	0	0	0
4 hrs	1+	0	0	2+	0	0
6 hrs	2+	0	0	2+	0	0
8 hrs	2+	0	0	2-3+	0	0
12 hrs	2-3+	0	0	3+	1+	0
24 hrs	3-4+	1+	0	3-4+	2+	0
30.5 hr	3-4+	1+	0	3-4+	2+	0
48 hrs	4+	2+	0	4+	2+	0
72 hrs	4+	2+	0	4+	2-3+	0
96 hrs	4+	2-3+	0	4+	2-3+	0
Day 7				3-4+	2+	0
Day 10				3-4+	2+	0

Table 27. Diamed results of weak B RBCs transformed at 4°C against anti-B.

Time	Btri (mg/mL)					
	washed			unwashed		
	0.6	0.3	0.15	0.6	0.3	0.15
2 hrs	0	0	0	0	0	0
4 hrs	0	0	0	1+	0	0
6 hrs	w+	0	0	1+	0	0
8 hrs	2+	0	0	2+	w+	0
12 hrs	2+	w+	0	2-3+	2+	0
24 hrs	4+	3+	2+	4+	3+	2+
30.5 hr	4+	2-3+	0	4+	2-3+	w+
48 hrs	4+	3+	1+	4+	3+	2+
72 hrs	4+	4+	2+	4+	4+	2+
96 hrs	4+	3-4+	2-3+	4+	3-4+	2-3+
Day 7				4+	2-3+	0
Day 10				4+	2+	0

Table 28. Diamed results of weak AB RBCs transformed at 4°C in block titre against anti-A.

Day	Btri (mg/mL)	Atri (mg/mL)					
		washed			unwashed		
		0.07	0.06	0.05	0.07	0.06	0.05
1	0.3	2+	1-2+	w+	2-3+	2+	1+
	0.2	2+	1-2+	0	2-3+	2+	1+
5	0.3	2+	1-2+	1+	2-3+	2+	1-2+
	0.2	2+	1-2+	w+	2-3+	2+	1-2+
8	0.3				2-3+	2+	2+
	0.2				2-3+	2+	1-2+

Table 29. Diamed results of weak AB RBCs transformed at 4°C in block titre against anti-B.

Day	Btri (mg/mL)	Atri (mg/mL)					
		washed			unwashed		
		0.07	0.06	0.05	0.07	0.06	0.05
1	0.3	3+	3+	2+	3+	3+	2-3+
	0.2	1+	1-2+	0	2+	2+	1-2+
5	0.3	2+	2+	1+	2+	2+	2+
	0.2	0	w+	vw	1+	w+	vw
8	0.3				2+	2+	2+
	0.2				1+	1+	0

Example 4 – Insertion efficiency of low temperature transformation of RBCs by A_{tri} and B_{tri} synthetic glycolipids

The post-transformation solutions (from A_{tri} 0.08 mg/mL, 0.05 mg/mL and 0.03 mg/mL, and B_{tri} 0.6 mg/mL, 20 μL) were added neat and in a 1:2 dilution to washed, packed RBCs (60 μL). The tubes were incubated in a 37°C waterbath for one hour, with mixing taking place every 15 minutes.

The transformed RBCs were washed 3x with PBS and then suspended in Cellstab at the appropriate concentration for serology testing.

Table 30. Tube serology

Pre-trans conc (mg/mL)	Score
Atri 0.08	0
1:2 of Atri 0.08	0
Atri 0.05	0
1:2 of Atri 0.05	0
Atri 0.03	0
1:2 of Atri 0.03	0
Btri 0.60	vw+
1:2 of Btri 0.60	0

The score given by the post-transformation solution (from the 0.08 mg/mL pre-transformation solution) is not even that of the 0.03 mg/mL transformation solution in the first pass (w+). These results indicate that >75% of the molecules are inserted into the RBC membrane on the first pass.

In addition, the post-transformation solutions were concentrated 20x and compared in parallel with the transformation solutions of known concentration. Only the post-transformation solutions derived from the 0.08 mg/mL Atri and 0.6 mg/mL Btri solutions were tested.

Post-transforrmation solutions (20 mL) were dialysed against de-ionised water for 2 days. The samples were left to dry in a fumehood for 10 days. At the end of this time they were transferred into a rotavapor flask and set on the rotavapor to rotate under vacuum with no heat overnight.

Warm bath to 40°C and dry samples. Wash over into smaller vessels with CM2:1 leaving significant amounts of dried cellular material. The CM2:1 washings were dried down, washed over again into test-tubes with CM2:1 and dried down. These samples were redissolved in 1 mL of 1 x PBS and used for transformation experiments. The cellular material in the bottom of the flasks was washed out with water into another set of tubes.

The post-transformation solutions (from Atri 0.08 mg/mL and Btri 0.6 mg/mL, 20 μL) were added to washed, packed RBCs (60 μL). In parallel, the transformation solutions (Atri 0.08 mg/mL, 0.05 mg/mL and 0.03 mg/mL, and Btri 0.6 mg/mL, 20 μL) were added to washed, packed RBCs (60 μL).

The tubes were incubated in a 37°C waterbath for one hour, with mixing taking place every 15 minutes. The transformed RBCs were washed 3x with PBS and then suspended in Cellstab at the appropriate concentration for serology testing.

Table 31. Diamed serology

conc (mg/mL)	Score
Atri 0.08	3+
Atri 0.05	2+
Atri 0.03	1+
From Atri 0.08	0
Btri 0.60	4+
From Btri 0.60	0

These results indicate that there are not enough molecules in the post-transformation solution even concentrated 20x to be detected by serology.

Example 5 – Transformation of RBCs by H_{trl} synthetic glycolipids

Mouse cells were transformed at 37°C for 1 hour.

Table 32. Anti-H reagents used for results in Table 29 and Table 30.

IgM
UEA
Bio-UEA

Table 33. Tube Serology.

	H Antisera			
Cells	IgM	UEA		Bio-UEA
		T = 0	T = 20	
Mouse RBCs (- control)	0	0	0	
Mouse RBCs + 0.01 mg/mL Htri	0			
Mouse RBCs + 0.05 mg/mL Htri	1+			
Mouse RBCs + 0.1 mg/mL Htri	3+			
Mouse RBCs + 0.25 mg/mL Htri	4+			1+
Mouse RBCs + 1 mg/mL Htri		2+		2+
Human O RBCs (+ control)	4+	1+	2/3+	4+

Table 33. Diamed

Cells	Score
Mouse RBCs + 0.01 mg/mL Htri	0
Mouse RBCs + 0.05 mg/mL Htri	0
Mouse RBCs + 0.1 mg/mL Htri	2+
Mouse RBCs + 0.25 mg/mL Htri	3+

Example 6 – Transformation of RBCs by filtered A_{tri} synthetic glycolipid

Some A_{tri} had been sterile-filtered through a 0.2 μm filter. To investigate whether transformation would be that same with this product, a comparative trial was done.

Table 34. Anti-A used for results presented in Table 32.

Manufacturer	Catalogue ref	Batch number	Expiry date
BioClone, OCD	Experimental reagent	01102	-

Table 35. Column agglutination of A RBCS transformed with varying concentrations of sterile-filtered vs normal A_{tri}.

Concentration (mg/mL)	Sterile syn-A	Normal syn-A
0.2	4+	4+
0.1	4+	3-4+
0.05	2-3+	2-3+
0.01	0	0
Control 37°C	0	
Control 25°C	0	

These results show no significant difference between the two groups. The fact that the sterile-filtered A_{tri} had a slightly stronger agglutination at 0.1 mg/mL than the normal A_{tri} shows that sterile filtration through a 0.2 μM filter did not remove molecules or change the composition or properties of the fluid to the point that transformation was affected.

Example 7 – Storage of transformed cells

To investigate whether storage at 4°C or 37°C changed the agglutination results of A_{tri} and natural glycolipid transformed O RBCs cells transformed cells, identified as Syn-A and Nat-A respectively, were divided in two and suspended to 5% in Cellstab. One set was stored at 4°C and the other was stored in a 37°C waterbath. The cells were tested

Table 36.

Time (hours)	Platform	Temp (°C)	A molecules Syn-A 0.1 mg/mL	Nat-A 1 mg/mL	10 mg/mL	Control
0	Tube		3+	0	1-2+	0
20	Column	4	4+	0	3+	0
		37	4+	0	3+	0
44	Column	4	4+		3+	0
		37	4+		3+	0

Example 8 - RBC transformation with A- and B-antigen synthetic glycolipid

The water soluble synthetic glycolipids designated A_{tri}-sp-Ad-DOPE (**I**), A_{tri}-sp_1sp_2-Ad-DOPE (**II**), A_{tri}-sp-Ad-DSPE (**III**), and B_{tri}-sp-Ad-DOPE (**VI**) were prepared according to the method described in Example 1 with necessary modifications.

Washed packed group O red blood cells (RBCs) (3 parts by volume) and the synthetic glycolipid solution (1 part by volume, varying concentrations) were added to an eppendorf tube. The tube was incubated in a 37°C waterbath for one hour, mixing every 15 minutes. The transformed RBCs were washed 3x with PBS and then suspended in Cellstab at the appropriate concentration for serology testing.

Tube serology and Diamed gel-card results for RBCs transformed with the different synthetic molecule constructs are provided in Table 37. Results for the stability of the RBCs transformed with the different synthetic glycolipids at different concentrations are provided in Tables 38 to 43.

A and B Antisera:

Antisera	Manufacturer	Batch
Albaclone anti-A	SNBTS	Z0010770 – D.O.E 12.12.04
Bioclone anti-A	Ortho Diagnostics	01102 – D.O.M 16.05.02
Albaclone anti-B	SNBTS	Z0110670 – D.O.E 12.12.04
Bioclone anti-B	Ortho Diagnostics	01103 – D.O.M 16.05.02

Table 37. Comparison of transformation of RBCs using A-antigen synthetic molecule constructs with different non-carbohydrate structures, made to different concentrations.

Synthetic	Conc mg/mL	A Antisera			
		Albaclone anti-A		Bioclone anti-A	
		Tube	Diamed	Tube	Diamed
A_{tri}-sp-Ad-DOPE (I)	0.25	n.d.	4+	n.d.	4+
	0.1	n.d.	4+/3+	n.d.	4+/3+
	0.05	w+	2+	2+	2+
	0.04	w+	n.d.	1+	n.d.
	0.03	0	n.d.	w+	n.d.
	0.02	0	n.d.	0	n.d.

	0.01	0	0	0	0
A_{tri}-sp-Ad-DSPE (III)	0.25	n.d.	0	n.d.	0
	0.1	n.d.	0	n.d.	0
	0.05	0	0	0	0
	0.04	0	n.d.	0	n.d.
	0.03	0	n.d.	0	n.d.
	0.02	0	n.d.	0	n.d.
	0.01	0	0	0	0
A_{tri}-sp_1sp_2-Ad-DOPE (II)	0.25	n.d.	4+	n.d.	4+
	0.1	n.d.	4+	n.d.	4+/3+
	0.05	0	3+	0	3+
	0.04	0	n.d.	0	n.d.
	0.03	0	n.d.	0	n.d.
	0.02	0	n.d.	0	n.d.
	0.01	0	0	0	0
Incubated control	–	0	n.d.	0	n.d.
Bench control	–	0	n.d.	0	n.d.

Abbreviations: n.d. Not determined

Table 38. Stability trial of RBCs transformed with A_{tri}-sp-Ad-DOPE (I) at high concentrations (1 mg/mL, 0.5 mg/mL and 0.25 mg/mL). Agglutination by manual tube serology.

Day	Cell storage solution	Albaclone anti-A			Bioclone anti-A		
		Concentration of Transformation Solution (mg/mL)					
		1	0.5	0.25	1	0.5	0.25
2	Alsevers	4+	4+	4+	4+°	4+°	4+°
	Cellstab	4+	4+	3+	4+°	4+°	4+°
10	Alsevers	3+	2+	2+	4+°	4+°	3+
	Cellstab	4+°	3+°	2+	4+°	4+°	4+°
17	Alsevers	4+	4+	4+	4+°	4+°	4+°
	Cellstab	4+	4+	4+	4+°	4+°	4+°
24	Alsevers	4+	4+	4+	4+	4+	4+
	Cellstab	4+	4+	4+	4+°	4+	4+

Abbreviations: ° splatter

Table 39. Stability trial of RBCs transformed with A_{tri}-sp-Ad-DOPE (I) at low concentrations (0.1 mg/mL, 0.05 mg/mL and 0.025 mg/mL). Agglutination by manual tube serology.

Day	Cell storage solution	Albaclone anti-A			Bioclone anti-A		
		Concentration of Transformation Solution (mg/mL)					
		0.1	0.05	0.025	0.1	0.05	0.025
2	Alsevers	3+/2+	1+	1+/w+	2+	2+/1+	1+
	Cellstab	3+/2+	2+	1+	3+/2+	3+/2+	2+
8	Alsevers	2+	1+	w+	3+/2+	2+	2+
	Cellstab	2+	1+/w+	vw	3+°	2+	1+
15	Alsevers	2+	1+	0	3+	2+	Vw
	Cellstab	4+	w+	0	4+	4+	1+
22	Alsevers	2+	2+	0	3+	2+	w+
	Cellstab	4+	4+	1+	4+	4+	1+
44	Alsevers	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Cellstab	4+	2+	w+	4+	2+	w+

Abbreviations: n.d. Not determined
° splatter

Table 40. Stability trial of RBCs transformed with A_{tri}-sp-Ad-DOPE (I) at high concentrations (1 mg/mL, 0.5 mg/mL and 0.25 mg/mL). Agglutination in Diamed gel-cards.

Day	Cell storage solution	Albaclone anti-A			Bioclone anti-A		
		Concentration of Transformation Solution (mg/mL)					
		1	0.5	0.25	1	0.5	0.25
2	Alsevers	4+	4+	4+	4+	4+	4+
	Cellstab	4+	4+	4+	4+	4+	4+
10	Alsevers	4+	4+	4+	4+	4+	4+
	Cellstab	4+	4+	4+	4+	4+	4+
17	Alsevers	4+	4+	4+	4+	4+	4+
	Cellstab	4+	4+	4+	4+	4+	4+
24	Alsevers	4+	4+	4+	4+	4+	4+
	Cellstab	4+	4+	4+	4+	4+	4+
45	Alsevers	4+	4+	4+	4+	4+	4+
	Cellstab	4+	4+	4+	4+	4+	4+
59	Alsevers	4+	4+		4+	4+	
	Cellstab	4+	4+	4+	4+	4+	4+
73	Alsevers						
	Cellstab	4+	4+	4+	4+	4+	4+
88	Alsevers						
	Cellstab	4+	4+	4+	4+	4+	4+

Where there were insufficient cells for testing, blank spaces have been left.

Table 41. Stability trial of RBCs transformed with A_{tri}-sp-Ad-DOPE (I) at low concentrations (0.1 mg/mL, 0.05 mg/mL and 0.025 mg/mL). Agglutination in Diamed gel-cards.

Day	Cell storage solution	Albaclone anti-A			Bioclone anti-A		
		Concentration of Transformation Solution (mg/mL)					
		0.1	0.05	0.025	0.1	0.05	0.025
2	Alsevers	4+	2+	0	4+	3+	1+
	Cellstab	4+	2+	0	4+	3+	1+
8	Alsevers	4+	3+	0	4+	4+	1+
	Cellstab	4+	3+	0	4+	4+	1+
15	Alsevers	4+	2+	0	4+	3+/2+	1+
	Cellstab	4+	4+	0	4+	4+	1+
22	Alsevers	4+	3+/2+	0	4+	3+	w+
	Cellstab	4+	4+	0	4+	4+	1+
29	Alsevers	4+	2+	0	4+	3+	w+
	Cellstab	4+	3+	0	4+	4+	2+
43	Alsevers	4+	3+	w+	4+	4+	2+
	Cellstab	4+	4+/3+	0	4+	4+	1+
50	Alsevers	4+	3+	w+	4+	4+	2+
	Cellstab	4+	3+	0	4+	4+	1+
57	Alsevers	4+	3+/2+		4+	4+	
	Cellstab	4+	3+	0	4+	3+	w+
63	Alsevers						
	Cellstab	4+/3+	2+	0	4+	3+	0
71	Alsevers						
	Cellstab	4+/3+	2+	0	4+	3+	0
86	Alsevers						
	Cellstab	4+/3+	2+	0	4+	3+	0

Where there were insufficient cells for testing, blank spaces have been left.

Table 42. Stability trial of RBCs transformed with B_{tri}-sp-Ad-DOPE (**VI**) at high concentrations (1 mg/mL, 0.5 mg/mL and 0.25 mg/mL). Agglutination by manual tube serology.

Day	Cell storage solution	Albaclone anti-B			Bioclone anti-B		
		Concentration of Transformation Solution (mg/mL)					
		1	0.5	0.25	1	0.5	0.25
2	Alsevers	3+	3+	2+	2+	1+	1+
	Cellstab	3+	2+	2+	2+	2+	1+
9	Alsevers	4+	4+	2+	4+	3+	2+
	Cellstab	4+	4+	3+	4+	4+	2+
16	Alsevers	4+	4+	3+	4+	4+	2+
	Cellstab	4+	4+	2+	4+	4+	2+
23	Alsevers	4+	4+	3+	4+	4+	3+
	Cellstab	4+	4+	3+	4+	4+	3+
30	Alsevers	3+	3+	2+	2+	2+	2+
	Cellstab	4+	3+	2+	3+°	3+°	2+
37	Alsevers	3+	2+	1+	3+	2+	1+
	Cellstab	3+	3+	2+/1+	4+°	3+	1+
44	Alsevers	4+	3+	1+	3+	3+	w+
	Cellstab	4+	4+	n.d.	4+	4+	‡
51	Alsevers	3+	3+	2+	4+	3+	2+
	Cellstab	4+	4+	n.d.	4+	4+	2+

Abbreviations: ° splatter

Table 43. Stability trial of RBCs transformed with B_{tri}-sp-Ad-DOPE (**VI**) at high concentrations (1 mg/mL, 0.5 mg/mL and 0.25 mg/mL). Agglutination in Diamed gel-cards.

Day	Cell storage solution	Albaclone anti-B			Bioclone anti-B		
		Concentration of Transformation Solution (mg/mL)					
		1	0.5	0.25	1	0.5	0.25
2	Alsevers	4+	4+	2+	4+	4+	2+
	Cellstab	4+	4+	2+	4+	4+	2+
9	Alsevers	4+	4+	2+	4+	4+	2+
	Cellstab	4+	4+	3+	4+	4+	3+
16	Alsevers	4+	4+	2+	4+	4+	1+
	Cellstab	4+	4+	3+	4+	4+	3+
23	Alsevers	4+	4+	3+	4+	4+	3+
	Cellstab	4+	4+	3+	4+	4+	3+
30	Alsevers	4+	4+	3+	4+	4+	3+
	Cellstab	4+	4+	3+	4+	4+	3+
37	Alsevers	4+	4+	3+	4+	4+	3+
	Cellstab	4+	4+	3+	4+	4+	3+
44	Alsevers	4+	4+	2+	4+	4+	3+
	Cellstab	4+	4+	3+	4+	4+	4+/3+
51	Alsevers	4+	4+	2+	4+	4+	3+
	Cellstab	4+	4+	3+	4+	4+	3+
58	Alsevers	4+		1+	4+		2+
	Cellstab	4+	4+	2+	4+	4+	2+
72	Alsevers	4+		2+	4+		3+
	Cellstab	4+	4+	3+/2+	4+	4+	3+
87	Alsevers						
	Cellstab	4+	4+/3+	1+	4+	4+/3+	2+/1+
116	Alsevers						
	Cellstab	4+	3+	0	4+	4+/3+	1+

Where there were insufficient cells for testing, blank spaces have been left.

Example 7 - Red Blood Cell Transformation with H-antigen synthetic glycolipids

The water soluble synthetic glycolipids designated H_{tri}-sp-Ad-DOPE (**VII**), H_{di}-sp-Ad-DOPE (**VIII**) and Galβ-sp-Ad-DOPE (**IX**) were prepared according to the method described in Example 1 with necessary modifications.

Washed packed mouse RBCs (3 parts by volume) and the synthetic glycolipid solutions (1 part by volume of varying concentrations) were added to an eppendorf tube. The tube was incubated in a 37°C waterbath for one hour, mixing every 15 minutes. The transformed RBCs were washed 3x with PBS and then suspended in Cellstab at the appropriate concentration for serology testing.

Tube serology and Diamed gel-card results for RBCs transformed with the different synthetic glycolipids are presented in Table 44. The results show that three sugars (H_{tri}) are required for detection by anti-H IgM.

Antisera	Manufacturer	Batch
Anti-H IgM	Japanese Red Cross	HIRO-75
UEA	Lorne Laboratories	11549E D.O.E. 06.2004
Bio-UEA	EY Labs	201105-2

Table 44. Comparison of transformation of RBCs using H-antigen synthetic molecule constructs with different glycotopes, made to different concentrations.

Synthetic	Conc mg/mL	H Antisera				
		IgM		UEA		Bio-UEA
		Tube	Diamed	Tube T0	Tube T20	Tube
H_{tri}-sp-Ad-DOPE (**VII**)	1	n.d.	n.d.	2+	n.d.	2+
	0.25	4+	3+	n.d.	n.d.	1+
	0.1	3+	2+	n.d.	n.d.	n.d.
	0.05	1+	0	n.d.	n.d.	n.d.
	0.01	0	0	n.d.	n.d.	n.d.
H_{di}-sp-Ad-DOPE (**VIII**)	0.25	0	n.d.	n.d.	n.d.	n.d.
	0.1	0	n.d.	n.d.	n.d.	n.d.
	0.05	0	n.d.	n.d.	n.d.	n.d.
	0.01	0	n.d.	n.d.	n.d.	n.d.
Galβ-sp-Ad-DOPE (**IX**)	0.25	0	n.d.	n.d.	n.d.	n.d.
	0.1	0	n.d.	n.d.	n.d.	n.d.
	0.05	0	n.d.	n.d.	n.d.	n.d.
	0.01	0	n.d.	n.d.	n.d.	n.d.
Human O cells	–	4+	n.d.	1+	2/3+	4+
Incubated control	–	0	n.d.	0	0	n.d.
Bench control	–	0	n.d.	n.d.	n.d.	n.d.

Abbreviations: n.d. Not determined

Example 8 – Insertion of Hdi-sp-Ad-DOPE (VIII) and Galß-sp-Ad-DOPE (IX) synthetic glycolipids into murine red blood cells

The water soluble synthetic glycolipids designated H_{dI}-sp-Ad-DOPE (**VIII**) and Galβ-sp-Ad-DOPE (**IX**) were prepared according to the method described in Example 1 with necessary modifications.

Murine RBCs were washed 3x in 1x PBS. 30µl of packed RBCs were combined with 30µl of H_{dI}-sp-Ad-DOPE (**VIII**), and 30µl of packed RBCs were combined with 30µl Galß-sp-Ad-DOPE (**IX**), respectively. Both synthetic molecule constructs were at a concentration of 1.0 mg/ml. 30µl of 1x PBS was added to 30µl of packed RBCs to act as the control group. Cells were incubated for 90 minutes in a 37°C shaking water-bath. RBCs were washed 3x in 1x PBS.

Three groups of packed RBCs were incubated with an equal volume of lectin UEA-1 for 30 minutes at room temperature. The lectin was prepared in 1x PBS at a concentration of 0.1 mg/ml. 50µl of a 3% cell suspension was spun for 15 seconds in an Immunofuge at low speed. Results were read by tube serology. The results are presented in Table 45. The results show that neither anti-H IgM nor UEA-1 detects two sugars (H_{dI}).

Antisera	Manufacturer	Batch
Biotest anti-H	Biotest AG	
UEA	EY Labs	201105-2

Table 45. Murine RBCs transformed with Galß-sp-Ad-DOPE or H_{di}-sp-Ad-DOPE, assessed by agglutination.

Cell Type	Inserted Molecule	UEA-1	Mouse IgM^H
Murine RBC	Galβ (1mg/ml)	0	n.d.
Murine RBC	H_{di} (1mg/ml)	0	0
Murine RBC	Control (PBS)	0	0
Human RBC	Control(PBS)	4+	3+

Abbreviations: n.d. Not determined

Example 9 - Attachment of Modified Embryos to Transformed Endometrial Cells

The ability to effect qualitative and quantitative differences in the cell surface antigens expressed by other cell types was investigated. The abilty to enhance the adhesion of embryos to endometrial cells was adopted as a model system. The synthetic molecule constructs may therefore be employed in the method of enhancing embryo implantation as described in international patent application no PCT/NZ2003/000059 (published as WO 03/087346).

- **Endometrial Cell Transformation**

Insertion of water soluble synthetic molecule construct

A single cell suspension of endometrial epithelial cells was prepared. The endometrial cells were washed 3x by resuspending in CMF HBSS and centrifuging at 2000 rpm for 3 minutes. The washed cell preparation was resuspended in 50µl of M2.

Micro-centrifuge tubes each containing a 50µl solution of 5M/ml endometrial cells were prepared. To separate tubes of endometrial cells 50 µl of synthetic glycolipids A_{tri}-sp-Ad-DOPE (**I**) or B_{tri}-sp-Ad-DOPE A (**VI**), or 50 µl M2 were added to the control cells. The cells were incubated for 90 minutes at 37°C on a mixer. The endometrial cells were washed 3x by resuspending in CMF HBSS media and centrifuging at 2000 rpm for 3 minutes. The washed cell preparation was resuspended in 50µl of M2.

Test For Insertion Using Fluorescent Probe:

50 µl of corresponding primary murine monoclonal antibody was added to each tube. Each tube was incubated at room temperature for 10 minutes. Cells were washed 3x in M2 media. 10 µl of mouse anti-IgG FITC was added to each tube. Tubes were incubated at room temperature in dark conditions for 10 minutes. Endometrial cells were mounted on glass slides and viewed under a fluorescence microscope.

Test for Direct Agglutination:

5 µl of each group of cells was placed onto separate microscope slides. To each 5µl drop of cells 5 µl of a corresponding antibody was added. The cells were gently mixed on the slide for 2 minutes. Agglutination was visualised under the microscope. The results are presented in Table 46.

Antisera	Manufacturer	
Bioclone anti-A	Ortho Diagnostics	01102 D.O.M. 16.05.02
Bioclone anti-B	Ortho Diagnostics	Developmental reagent

Table 46. Endometrial cells transformed with A_{tri}-sp-Ad-DOPE (**I**) or B_{tri}-sp-Ad-DOPE A (**VI**), as visualised using fluorescence.

Cell Type	Inserted Antigen	1° antibody	Fluorescence score after incubation with IgFITC Probe	Agglutination reaction by microscopic visualisation
Endometrial cells	A_{tri} (1mg/ml)	Anti-A Bioclone	4+	4+
Endometrial cells	B_{tri} (1mg/ml)	Anti-B Bioclone	1+	3+
Endometrial cells	Control (M2 media)	Anti-A Bioclone	0	0

- **Embryo Modification**

Insertion of water soluble synthetic molecule construct:

The embryo zona pellucida was removed by treating embryos with 0.5% pronase in a 37°C oven for 6 minutes or until all zonas were removed. Micro-drops were prepared by adding 5µl of synthetic glycolipid A_{tri}-sp-Ad-DOPE (**I**) or B_{tri}-sp-Ad-DOPE (**VI**), at a concentration of 1 mg/mL to a 45µl drop of M2 media overlaid with mineral oil. All embryo groups were incubated in the 50µl micro-drops for 1 hour at 37°C. Embryos from experimental and control groups were washed 3x with M2 media.

Test for Insertion:

Embryos from experimental and control groups were placed into a micro-drop of corresponding antibody and incubate for 30 min at 37°C. Embryos from experimental and control groups were washed 3x with M2 media.

Embryos from all experimental and control groups were placed into micro-drops of anti-mouse Ig FITC (1:50 dilution anti-mouse Ig FITC in M2) and incubated for 30 min at 37°C. Embryos from experimental and control groups were washed 3x with M2 media. Embryos were mounted on microscope slides in a 5µl drop of M2 and the drops overlaid with oil.

The slides were viewed under a fluorescence microscope. Results are presented in Tables 47 and 48. The negative result for transformation with B_{tri}-sp-Ad-DOPE (**VI**) is attributed to a lack of 1° antibody sensitivity.

Table 47. Embryos transformed with A_{tri}-sp-Ad-DOPE (I) as visualised using fluorescence.				
Cell Type	Inserted Antigen	1° antibody	Fluorescence score after incubation with IgFITC Probe	Embryo Morphology 24hr post insertion
Embryos	A_{tri}	Anti-A Bioclone	2+/1+	Appeared viable
Embryos	Control	Anti-A Bioclone	0	Appeared viable

Table 48. Embryos transformed with A_{tri}-sp-Ad-DOPE (I) or B_{tri}-sp-Ad-DOPE (VI), as visualised using fluorescence.				
Cell Type	Inserted Antigen	1° antibody	Fluorescence score after incubation with IgFITC Probe	Embryo Morphology 24hr post insertion
Embryos	A_{tri}	Anti-A Bioclone	2+	n.d.
Embryos	B_{tri}	Anti-B Bioclone	0	n.d.
Embryos	Control (M2 media)	Anti-A Bioclone	0	n.d.

Abbreviations: n.d. Not determined

Enhanced Attachment Transformed Endometrial Cells to Modified Embryos

Modified embryos (BioG-Avidin-BioIgG^B and BioG-Avidin-BioIgM^A) were prepared in accordance with the methods described in the specification accompanying the international application no. PCT/NZ03/00059 (published as WO03087346)

Two concave glass slides were prepared, one with two wells of synthetic molecule construct A_{tri}-sp-Ad-DOPE (I) inserted endometrial cells and the other with two wells of synthetic molecule construct B_{tri}-sp-Ad-DOPE (VI) inserted endometrial cells.

The two groups of embryos were transferred to each of the concave glass slides:

Slide 1 A_{tri}/IgG^B embryos
A_{tri}/IgM^A embryos

Slide 2 B_{tri}/IgG^B embryos
B_{tri}/IgM^A embryos

The embryos were surrounded with endometrial cells. The wells were covered with mineral oil and incubated for 15 minutes at 37°C. Using a wide bore handling pipette each group of embryos were carefully transferred to a fresh drop of M2 media. The embryos were gently washed. The embryos were gently transferred into 2µL of M2 media on a marked microscope slide. Each drop was overlaid with mineral oil

Under a central plane of focus on an Olympus microscope the number of endometrial cells

adhered to the embryos in each group was assessed. The number of cells adhered to each embryo was recorded. Results are presented in Table 49.

Table 49. Endometrial cells transformed with A_{tri}-sp-Ad-DOPE (**I**) or B_{tri}-sp-Ad-DOPE (**VI**), and embryos modified with BioG-Avidin-BioIgG^B or BioG-Avidin-BioIgM^A. Assessment by attachment of endometrial cells to embryos.

Cell Type	Transformed endometrial cells	Modified embryos	Average number of endometrial cells attached to modified embryos
Endometrial cells	A_{tri}-sp-Ad-DOPE (**I**)	BioG-Avidin-BioIgG^B	2.3
		BioG-Avidin-BioIgM^A	7.25
Endometrial cells	B_{tri}-sp-Ad-DOPE (**VI**)	BioG-Avidin-BioIgG^B	6.7
		BioG-Avidin-BioIgM^A	3.4

Where in the foregoing description reference has been made to integers or components having known equivalents then such equivalents are herein incorporated as if individually set forth. For example, where in the Examples reference has been made to "A_{tri}" and "B_{tri}" the synthetic molecule constructs A_{tri}-sp-Ad-DOPE (**I**) and B_{tri}-sp-Ad-DOPE (**VI**), respectively, have been used. This is not intended to imply that other synthetic molecule constructs of the invention could not be used.

Although the invention has been described by way of example and with reference to possible embodiments thereof it is to be appreciated that improvements and/or modification may be made thereto without departing from the scope or spirit of the invention.

REFERENCES

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BALDWINS

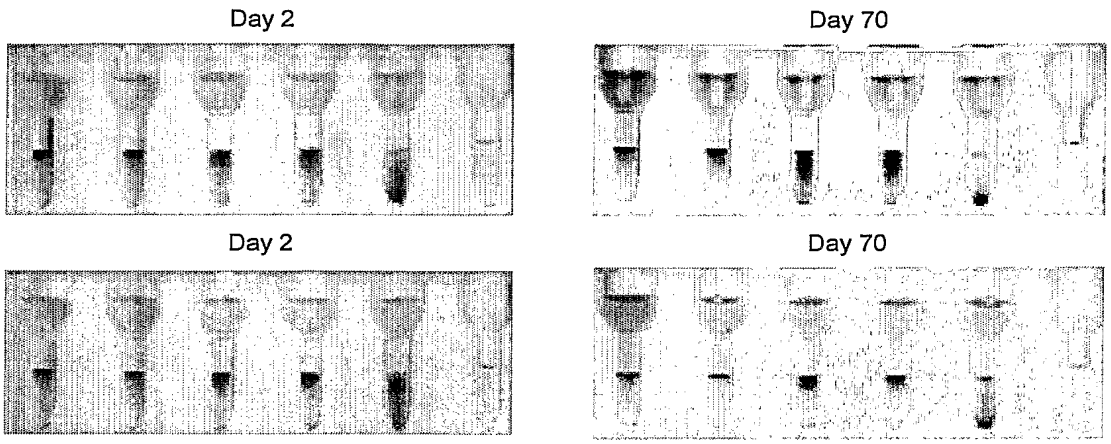

FIGURE 1

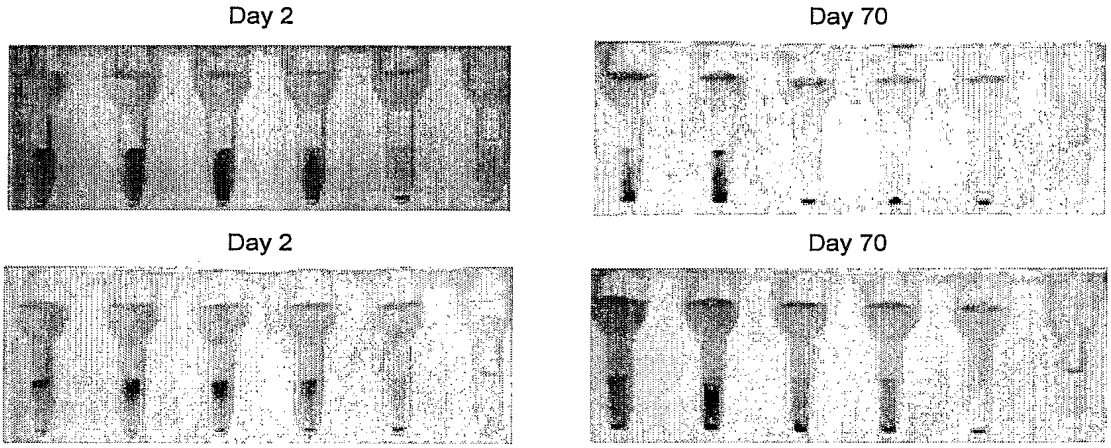

FIGURE 2

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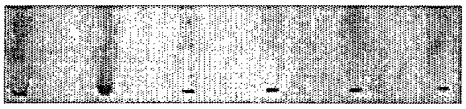
2 hours

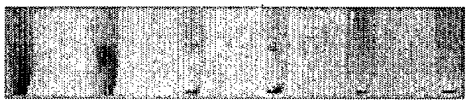
All cells negative

4 hours

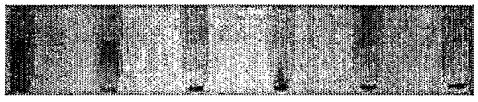


6 hours

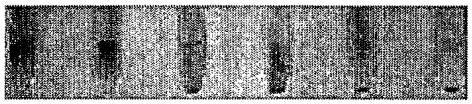
8 hours



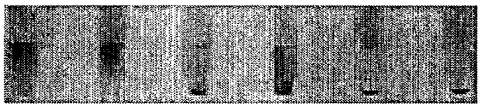
12 hours



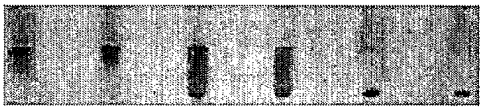
24 hours



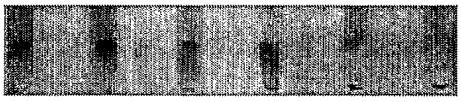
30.5 hours



48 hours



72 hours



96 hours

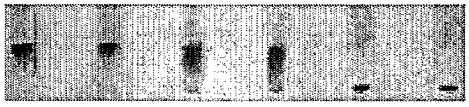


FIGURE 3

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Day 7

Day 10

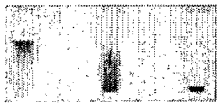

FIGURE 4

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2 hours

All cells negative

4 hours

6 hours

8 hours

12 hours

24 hours

30.5 hours

48 hours

72 hours

96 hours

FIGURE 5

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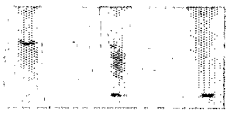
Day 7



Day 10

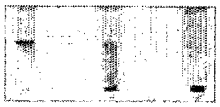
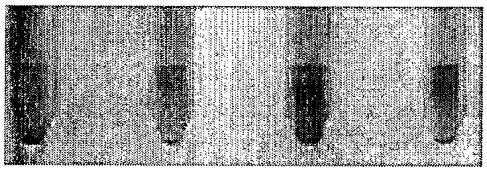


FIGURE 6

Day 1



Day 5

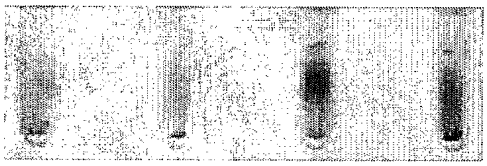


FIGURE 7

Day 8

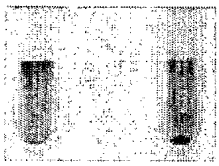


FIGURE 8

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Day 1

Day 5

FIGURE 9

Day 8

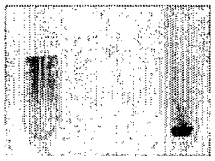
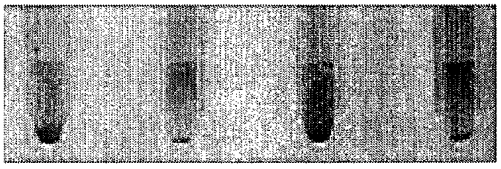


FIGURE 10

Day 1



Day 5

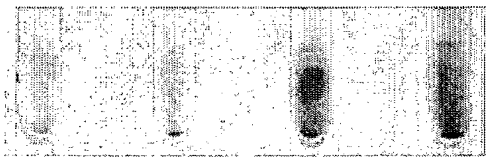


FIGURE 11

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Day 8

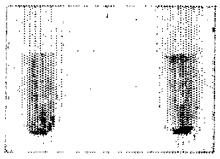
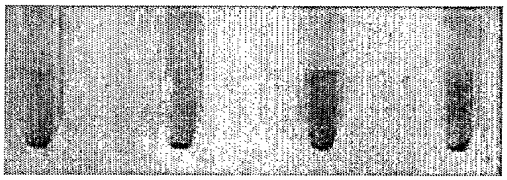


FIGURE 12

Day 1



Day 5

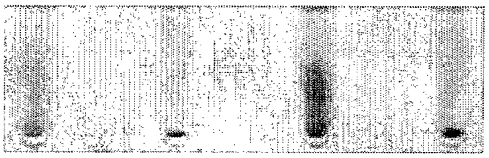


FIGURE 13

Day 8

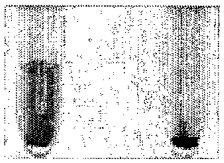


FIGURE 14

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Day 1

Day 5

FIGURE 15

Day 8

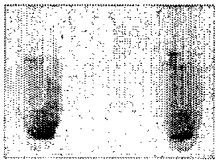
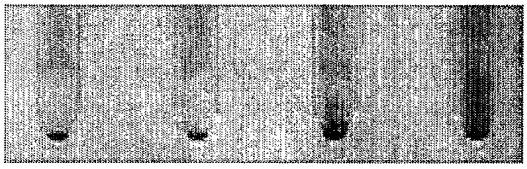


FIGURE 16

Day 1



Day 5

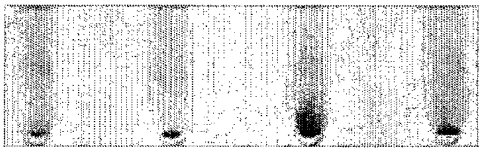


FIGURE 17

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Day 8

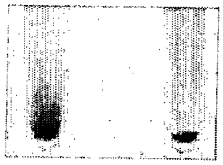

FIGURE 18

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